

**USE OF PEA PROTEIN-POLYSACCHARIDE MICROCAPSULES
AS A DELIVERY SYSTEM FOR PROBIOTIC BACTERIA;
TESTING UNDER SIMULATED GASTRIC CONDITIONS
AND IN ANIMAL MODEL SYSTEMS**

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ABSTRACT

Pea protein-polysaccharide microcapsules (2.0 % (w/v) of pea protein isolate (PPI), mixed with either sodium alginate, *iota*-carrageenan or gellan gum (0.5 % (w/v)), containing *Bifidobacterium adolescentis* were tested in a series of *in vitro* survival experiments to evaluate the ability of microcapsules to protect the bacterium under simulated stomach conditions, as well as their ability to release the encapsulated bacteria under simulated conditions of the lower gut. All tested capsule formulations provided significant protection for bifidobacteria relative to non-encapsulated bacteria. PPI-alginate and PPI-*iota*-carrageenan microcapsules released 70-79 % of encapsulated bacteria, with higher cell numbers being released from freeze-dried capsules. The number of released cells from PPI-gellan gum microcapsules was ~ 26-30 % lower. A rat feeding study was conducted with the test bacterium encapsulated in PPI-alginate. *Bifidobacterium adolescentis*-specific PCR and qPCR analyses confirmed the presence of DNA from this species in animal fecal samples, but only during the period of capsule intake.

Using a mixture of two lactobacilli (a combination of *Lactobacillus rhamnosus* strain R0011 and *Lactobacillus helveticus* strain R0052; Lacidofil[®], Lallemand Health Solutions, Montreal, QC, Canada) known to relieve *Citrobacter rodentium* colitis in mice, we assessed the impact of immobilization of these cells (PPI-alginate) using this animal disease model. The main objective was to determine whether PPI-alginate encapsulation matrix interfered with the ability of probiotic bacteria to reduce the symptoms of colitis. Animals were fed rodent AIN93G diets (control) or AIN93G diets supplemented with either freeze dried or microencapsulated bacterial cells. A second control consisting of PPI-alginate microcapsules only was also fed to mice. Half of the animals in each treatment group were infected with *C. rodentium*. Daily monitoring of disease symptoms, analyses of histopathological changes in intestinal tissues, cytokine expression levels, and bacterial densities in fecal samples were assessed. Additionally, 16S rRNA gene sequencing of the mucosal microbial communities in the distal colon was conducted. Infection of mice with *C. rodentium* led to a marked progression of infectious colitis, as evidenced from symptomatic and histopathological data, changes in cytokine expression levels, and alteration of composition of mucosa-associated bacterial communities. Probiotic administration affected disease markers in the cecum but not in the colon, but had no significant impact on cytokine profiles in infected animals. Based on cytokine expression analyses and histopathological data, it was evident that encapsulation contributed to the development of inflammation and worsened symptoms in this experimental animal model. In

general, the results of this study suggest that even though the tested encapsulation materials are generally recognized as safe, they may in fact contribute to the development of an inflammatory response in certain animal disease models when administered in the capsule form.

In the last study, PPI–alginate microcapsules with or without a chitosan coating and containing *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 were produced by extrusion and tested for survivability during storage and under *in vitro* gastrointestinal conditions. Storage at 4°C had no negative impact on capsule protective ability in both types of capsules. PPI-alginate microcapsules with chitosan containing showed much higher bacterial survival counts than microcapsules without extra coating during challenge tests even after the storage period.

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LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|-------------|--|
| ANOVA | Analysis of variance |
| APW | Alkaline peptone water |
| ATCC | American Type Culture Collection |
| C | <i>Citrobacter rodentium</i> infection |
| CFU | Colony-forming unit |
| CLSM | Confocal laser scanning microscope |
| cm | Centimeter |
| d | Day |
| DMEM | Dulbecco's Modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |
| E | Encapsulation |
| g | Gram |
| G | Gauge |
| GIT | Gastrointestinal tract |
| h | Hour |
| <i>Il4</i> | Interlekin 4 |
| <i>Il17</i> | Interleukin 17 |
| <i>Infγ</i> | Intergeron gamma |
| kDa | Kilodalton |
| LAB | Lactic acid bacteria |
| M | Molar |
| mg | Milligram |
| min | Minute |
| mM | Micromolar |
| mm | Millimeter |
| MRS | de Man, Rogosa and Sharpe medium |
| N | Normality |
| NGS | Next-generation sequencing |
| OTU | Operational taxonomic unit |
| P | Probiotic |

| | |
|--------------|---------------------------------------|
| PA | Pea protein isolate-alginate |
| PAC | Pea protein isolate-alginate-chitosan |
| pI | Isoelectric point |
| PPI | Pea protein isolate |
| rpm | Revolutions per minute |
| s | Second |
| SGF | Simulated gastric fluid |
| SIF | Simulated intestinal fluid |
| SSJ | Synthetic stomach juice |
| TFG- β | Transforming growth factor beta |
| v/v | Volume by volume |
| w/v | Weight by volume |
| w/w | Weight by weight |
| μ L | Microliter |

1 INTRODUCTION

1.1 Overview

This research focuses on evaluating materials for microencapsulating probiotic bacteria to provide protection from the detrimental conditions of the upper gastrointestinal tract (GIT) and their controlled release under simulated intestinal conditions. According to the current definition of the Food and Agriculture Organization/World Health Organization, probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Probiotic bacteria used in the research were strains of lactobacilli and bifidobacteria. Lactobacilli are used most often in probiotic research. Among the purported beneficial effects, these bacteria can improve digestion, absorption and availability of nutrients (Wallace *et al.*, 2011). Furthermore, lactobacilli are capable of hydrolyzing compounds that limit the bioavailability of minerals, like tannin and phytate, due to tannin acylhydrolase and phytase activities (Turpin *et al.*, 2010). The purported benefits of bifidobacteria include, but are not limited to, reducing the number of harmful organisms in the intestine, producing antimicrobial substances and stimulating the body's immune response (Holzapfel and Schillinger, 2002; Wen *et al.*, 2012). The highly-acidic stomach environment reduces the number of viable probiotic cells; hence, the search for novel protective strategies remains ongoing. Biopolymers of plant and bacterial origin are reported to be successful candidates for this purpose as they are generally recognized as safe, and have gel-forming capabilities. Biopolymer-based microcapsules thus offer a potential strategy for delivery of viable probiotic bacteria during passage through the harsh GIT environment, with subsequent benefits to human health.

Specifically, three hydrocolloid polymers, combined with pea protein isolate (PPI), were tested for their ability to protect bacterial cells: sodium alginate, *iota*-carrageenan and gellan gum. All of these biopolymers are widely used in food production, commercially available at a reasonable cost and form gels in the presence of calcium ions (Champagne and Fustier, 2007; Chávarri *et al.*, 2010; Luna *et al.*, 2010).

According to previous studies (Kotikalapudi *et al.*, 2010; Klemmer *et al.*, 2011), PPI was shown to enhance the survival of bacterial cells immobilized inside PPI-alginate microcapsules. Since carrageenans and gellan gum have also been used for the delivery of various substances into the GIT, the protective properties of PPI to enhance survival within these matrices was also examined. Anaerobic *Bifidobacterium adolescentis* was used for encapsulation to test microcapsule composition both *in vitro* and *in vivo*.

Subsequently, the effects of the chosen microcapsule composition on the activity of probiotics in an animal model of a human disease (e.g., enhancing or interfering with probiotic effects) were examined. The goal was to evaluate the ability of PPI-alginate microcapsules to safely and effectively deliver probiotics in a mouse colitis model system and to study the effects of capsule composition on both disease symptoms and the microbiome, using various health-related (cytokine product, inflammation, etc.) and microbial parameters (bacterial numbers). A final study focused on improving capsule formulation by incorporating chitosan into the matrix. The survival of probiotics in PPI-alginate microcapsules with or without chitosan coating was investigated during storage and in simulated gastrointestinal environment.

1.2 Objectives

The overall objectives of this research were: i) to develop a method for producing protective microcapsules containing probiotic bacteria, utilizing a complex of PPI and one of three biopolymers - sodium alginate, *iota*-carrageenan or gellan gum, ii) to investigate the effect of the microcapsule composition on the survival of bacteria during exposure to the simulated conditions of the upper GIT and on the release of bacterial cells from microcapsules under simulated intestinal conditions; iii) to determine, using an animal model, the numbers of probiotic bacterium delivered to the lower GIT, as well as any impact that the capsular materials may have on the host; and iv) to determine the impact of encapsulation materials on the ability of probiotic bacteria to restore microbial balance in the animal GIT, resulting from pathogenic infection.

1.3 Hypotheses

The following hypotheses were tested as part of this research:

- The addition of PPI to hydrocolloids (alginate, *iota*-carrageenan, gellan gum) will enhance the survival of *B. adolescentis* immobilized within microcapsules (compared to planktonic bacteria) under simulated upper GIT conditions and allow the release of the entrapped

bacteria under simulated intestinal conditions;

- The selected microcapsule composition will allow delivery of *Bifidobacterium adolescentis* into the lower GIT of laboratory animals at detectable concentrations;
- The encapsulation of probiotics, selected *in vitro* for their antagonistic properties against a pathogen, will not interfere with their ability to treat or alleviate the symptoms of induced infectious disease in an animal model;
- The additional improvement (outer chitosan layer) will enhance protective properties of microcapsules.

2 LITERATURE REVIEW¹

2.1 Microbial ecology of the human gastrointestinal tract

The human intestinal microbiota is a complex ecosystem with considerable impact on human health and well-being, contributing to maturation of the immune system and providing a direct barrier against pathogen colonization (Doré and Corthier, 2010). It consists of bacteria, archaea, some protozoa, anaerobic fungi and different bacteriophages and viruses, and it has been estimated that more than 1000 species of microbes inhabit the human intestine (Tuohy *et al.*, 2012). The presence of a great number of microbes (up to 5×10^{11} bacterial cells per gram of intestinal contents) suggests strong regulatory effects on the human host, and recent findings suggest that gut microbiota can have a considerable impact on both our weight and mood (Duca *et al.*, 2014; Naseribafrouei *et al.*, 2014). The composition and function of human microbial populations associated with various body sites have been studied with the help of metagenomic tools as part of two recent initiatives – the NIH Human Microbiome Project (HMP) and the European Metagenomics of the Human Intestine (metaHIT) project (NIH HMP Working Group *et al.*, 2009; Dusko Ehrlich *et al.*, 2011).

Bacteria that initially colonize the lower GIT of an infant are facultative anaerobes, such as *Escherichia coli* and *Streptococcus spp.* These species metabolize oxygen in the gut, thereby creating anaerobic conditions. Subsequent colonization largely depends on food profile and environmental factors (i.e., sanitary conditions). After the full formation of the gastrointestinal microflora, its composition has been shown to include such genera as *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Lactobacillus*, *Fusobacterium* and various Gram-positive cocci (Fooks *et al.*, 1999; Wallace *et al.*, 2011).

Within the GIT, the microbiota perform various functions, such as digestion of nutrients and maturation of intestinal epithelial cells. Studies on mice have shown a number of significant effects of microbiota on the host. In ex-germ-free, reconvventionalized mice, their intestinal epithelium was thicker, short-chain fatty acids were produced at significantly higher concentrations, and there was a

¹Chapters 2.1-2.3: Varankovich, N. V., Nickerson, M. T., and Korber, D. R. (2015). Probiotic-based strategies for therapeutic and prophylactic use against multiple gastrointestinal diseases. *Front Microbiol* 685, 1-14.

normal level of immunological activity present, compared to germ-free animals (Aureli *et al.*, 2011). Microbes also have the ability to affect physiologic parameters, providing systemic effects on blood lipids and generally influencing the immune system, as well as inhibiting harmful bacteria (Mikelsaar, 2011). Pathogen inhibition by human intestinal microbiota may provide significant human health benefits through protection against infection as a natural barrier against pathogen exposure in the gastrointestinal tract (Wallace *et al.*, 2011). Factors such as food contamination by pathogens, as well as the high load of antibiotics in animal feed, resulting in the presence of these substances in animal-based food products, can influence the microbial ecology of human gastrointestinal tract (Sapkota *et al.*, 2007). Using molecular genetic tools, it has been shown that antibiotics could induce significant alterations in the dominant colonic flora that are not detectable using bacteriological (culture-based) techniques, with effects lasting for up to two months (Mangin *et al.*, 1999). Several more specific disorders involve disruption of the human microflora ecology: acute gastroenteritis, *Clostridium difficile* infection (CDI), necrotising enterocolitis in neonates, irritable bowel syndrome and *Helicobacter pylori* infection (Kotzampassi and Giamarellos-Bourboulis, 2012). Probiotics are currently being examined for their potential treatments of the aforementioned disorders.

2.2 Probiotic bacteria

As defined by the Food and Agriculture Organization/World Health Organization, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). The most common probiotics include representatives of lactobacilli, enterococci, bifidobacteria and yeasts (Table 2.1). In addition, bacterial mixtures may potentially be used to achieve the complex beneficial effect of probiotics (Caballero-Franco *et al.*, 2007).

Presumed health benefits of probiotics include reducing harmful organisms in the intestine, producing antimicrobial factors and stimulating the body's immune response (Collado *et al.*, 2007; Foligné *et al.*, 2010; Konieczna *et al.*, 2013). Some of the potential beneficial effects of probiotics, seen in animal studies (e.g., lowering of cholesterol level) are yet to be substantiated by well-controlled clinical trials. However, there are a growing number of studies providing data on effects of probiotic bacteria on the human immune system and on microflora of the gastrointestinal tract (Holzapfel and Schillinger, 2002; Foligné *et al.*, 2007; Verdú *et al.*, 2009; Wen *et al.*, 2012).

Table 2.1. Microorganisms with reported probiotic potential.

| Probiotic group | Species | Details of a representative study | Reference |
|-----------------|------------------------|--|---|
| Bifidobacteria | <i>B. animalis</i> | Fermented oak milk with <i>B. animalis</i> subsp. <i>lactis</i> BB-12 [®] ; double-blind randomized placebo-controlled clinical trial; n = 209; 35 % improvement in bowel movement. | Pitkala <i>et al.</i> , 2007 (See Jurgensen <i>et al.</i> (2014) for the review) |
| | <i>B. breve</i> | <i>B. breve</i> M16-V powder with or without starch; F344/Du rat pups; n = 46; downregulation of the expression of inflammatory molecules. | Shimakawa <i>et al.</i> , 2003 |
| | <i>B. adolescentis</i> | <i>B. adolescentis</i> ATCC 101; female germ-free rats; n = 30; significant modulation of both systemic and the intestinal immune response to <i>Bacteroides thetaiotaomicron</i> DSMZ 2079. | Scharek <i>et al.</i> , 2000 |
| | <i>B. longum</i> | <i>B. longum</i> , isolated from human GIT; double-blind randomized placebo-controlled clinical trial; n=29; significant inhibitory effect on viral gastroenteritis symptoms. | Lee <i>et al.</i> , 2014b |
| | <i>B. infantis</i> | <i>B. infantis</i> 35624 was administered by gavage; C57BL/6 mice; n=64; decrease in the severity of dextran sulfate sodium-induced colitis, immunomodulation. | Konieczna <i>et al.</i> , 2013 |
| Lactobacilli | <i>L. acidophilus</i> | <i>L. acidophilus</i> - SDC, administered in capsules; double-blind randomized placebo-controlled clinical trial; n = 40; 23.6 % reduction (compared to placebo) in the severity (pain, discomfort) of Irritable Bowel Syndrome. | Sinn <i>et al.</i> , 2008 |

Table 2.1. Microorganisms with reported probiotic potential. Continued.

| Probiotic group | Species | Details of a representative study | Reference |
|-----------------|---|---|------------------------------------|
| | <i>L. fermentum</i> <i>L. amylovorus</i> | Microencapsulated bacteria; double-blind randomized placebo controlled clinical trial; n = 28, obese adults; <i>L. fermentum</i> – 3.0 % loss in total fat mass, <i>L. amylovorus</i> – 4.0 % loss in total fat mass, a significant reduction in the abundance of Clostridial cluster IV. | Omar <i>et al.</i> , 2013 |
| | <i>L. rhamnosus</i> | <i>L. rhamnosus</i> GG powder; double-blind randomized clinical trial; n = 559; decrease in frequency and duration of acute watery diarrhea. | Basu <i>et al.</i> , 2009 |
| | <i>L. paracasei</i> | <i>L. paracasei</i> ST11, lyophilized form; double-blind randomized placebo-controlled clinical trial; n = 230, male infants and young children; significant benefit in the management of children with nonrotavirus-induced diarrhea. | Sarker <i>et al.</i> , 2005 |
| | <i>L. johnsonii</i> | <i>L. johnsonii</i> La1 in dietary product; double-blind randomized placebo-controlled clinical trial; n = 326, children, found positive for <i>Helicobacter pylori</i> ; significant decrease in $\delta^{13}\text{CO}_2$ above baseline values (outcome of a test for <i>H. pylori</i>). | Cruchet <i>et al.</i> , 2003 |
| | <i>L. reuteri</i> | <i>L. reuteri</i> SD 2112; randomized controlled clinical trial; n = 40, infants and children; significant decrease in diarrhea symptoms. | Shornikova <i>et al.</i> , 1997 |
| | <i>Pediococcus pentosaceus</i> | <i>P. pentosaceus</i> NB-17; mouse spleen cells were co-cultivated with heat-killed bacteria; <i>in vitro</i> investigation of the production of cytokines; effective stimulation of immune activities and allergic inhibitory effects. | Jonganurakkun <i>et al.</i> , 2008 |

Table 2.1. Microorganisms with reported probiotic potential. Continued.

| Probiotic group | Species | Details of a representative study | Reference |
|-----------------|--------------------------------|---|--|
| | <i>Oenococcus oeni</i> | <i>O. oeni</i> 9115; female BALB/c mice with 2, 4, 6-trinitrobenzene sulfonic acid-induced experimental colitis; n = 20; significant decrease in severity of colitis. Several <i>O. oeni</i> strains were able to modulate the immune response of immunocompetent cells <i>in vitro</i> . | Foligné <i>et al.</i> , 2010 |
| Enterococci | <i>E. durans</i> | <i>E. durans</i> LAB18s; <i>in vitro</i> study; antimicrobial activity, antioxidant ability, evidenced in both culture supernatants and intracellular extracts; resistance to acidic conditions (pH 3) and bile salts. | Pieniz <i>et al.</i> , 2014 |
| | <i>E. faecium</i> | <i>E. faecium</i> MMRA; <i>in vitro</i> study; genes, coding enterocins A, B, P and X; high survival rates under stress caused by acidic pHs (2-5) or bile salts (0.3 %), and a high adhesive potential. | Rehaïem <i>et al.</i> , 2014 |
| | <i>E. faecalis</i> | <i>E. faecalis</i> UGRA10; <i>in vitro</i> study; production of AS-48 enterocin; ability to form biofilms and to adhere to Caco 2 and HeLa 229 cells. | Cebrián <i>et al.</i> , 2012 |
| | <i>E. lactis</i> | <i>E. lactis</i> IITRHR1 was administered by gavage; male Wistar rats with acetaminophen-induced hepatotoxicity; n = 42; pretreatment with the bacterium lowered the level of biomarkers of hepatotoxicity in serum; significant increase in the level of antioxidant; modulation of key apoptotic/anti-apoptotic proteins (cytochrome-c, Bcl2, Bax, expression of caspases). | Sharma <i>et al.</i> , 2012 |
| Yeasts | <i>Saccharomyces boulardii</i> | Granulated <i>S. boulardii</i> ; double-blind randomized placebo-controlled clinical trial; n = 200, children with acute diarrhea; significant decrease in severity of symptoms and duration of hospital stay. | Kurugol and Koturoglu, 2005 (See Kelesidis and Pothoulakis (2010) for the review) |

Increasingly, reports of the human/animal microbiome playing a central role in other key aspects of health functionality are emerging, including beneficial impacts on the treatment of metabolic disorders, such as obesity and type II diabetes, improvement of bowel function in patients with colorectal cancer, potential cognitive and mood-enhancing benefits, antidepressant and anxiolytic (antianxiety) activity (Desbonnet *et al.*, 2008; Bravo *et al.*, 2011; DiBaise *et al.*, 2012; Lee *et al.*, 2014a; Owen *et al.*, 2014). The latter anxiolytic effect has even led to the emergence of the new term, “psychobiotic”, coined by Dinan *et al.* (2013) as “a live organism that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric illness”.

Products containing probiotic bacteria generally include supplements and foods. Live probiotics are commonly-available in fermented dairy products and probiotic-fortified foods. These bacteria are added into numerous foods and beverages, ranging from yoghurts to breakfast cereals. There are also tablets, capsules, powders and sachets containing probiotics in freeze-dried form. Functional foods, defined as food preparations with various health-related properties, often include bacterial strains with declared probiotic properties (Turrioni *et al.*, 2011). The scientific interest towards probiotics is growing exponentially: the search for published papers featuring keyword “probiotic” in NIH PubMed database revealed 7265 articles for the period from 2000 to 2010, with 953 of them being clinical trials. Within the following 5 years (up to May 20th 2015), the frequency of publications doubled with 7979 papers being published during that period, including 778 clinical trials.

2.2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are Gram-positive, non-spore forming cocci, coccobacilli or rods, which generally have non-respiratory (fermentative) metabolism and lack true catalase. Unlike bifidobacteria, which are active in the lower colon, lactobacilli are generally found in the upper GIT (Turrioni *et al.*, 2011). LAB are also normal members of the human microflora, and found in the oral cavity, the small intestine, and the vaginal epithelium, where they are thought to play beneficial roles (Gomes and Malcata, 1999). Among the purported beneficial effects, lactobacilli have been claimed to improve digestion, absorption and availability of nutrients (Wallace *et al.*, 2011). Furthermore, LAB are capable of hydrolyzing compounds that limit the bioavailability of minerals, like tannin and phytate, due to tannin acylhydrolase and phytase activities (Turpin *et al.*, 2010). In addition, it was shown that some lactobacilli strains could improve mineral absorption in Caco-2 cells and improve

the nutritional status of the host by producing B-group vitamins. More recently, the role of lactobacilli in energy homeostasis, particularly in obese patients, has been the object of an increased interest (Guo *et al.*, 2010; Mikelsaar, 2011). A further potential impact of LAB is their ability to inhibit or kill *Helicobacter pylori*, which is now regarded as the major cause of gastritis and peptic ulcers and is a risk factor for gastric malignancy (Hamilton-Miller, 2003). In addition, both *Lactobacillus spp.* and *Bifidobacterium spp.* reportedly reduce the side effects of *Helicobacter pylori* eradication therapy (Canducci *et al.*, 2002).

Pediococci are also related to the LAB group and are utilized in industrial fermentations of foods and silage (Raccach, 2014). Pediocin-producing *Pediococcus spp.* strains are of potential interest to food safety (Raccach, 2014), with three of them potentially possessing probiotic properties – *P. pentosaceus*, *P. parvulus* and *P. acidilactici*. Osmanagaoglu *et al.* (2010) comprehensively studied the potential of a human *P. pentosaceus* isolate for probiotic use, and reported that the strain produced an anti-*Listerial* bacteriocin, had excellent autoaggregation characteristics and was also able to co-aggregate with *Salmonella enterica* serotype Typhimurium and enterotoxigenic *Escherichia coli* (Osmanagaoglu *et al.*, 2010). Antagonistic activity against *Listeria monocytogenes* was also discovered in *P. acidilactici* (Guerra and Pastrana, 2002). Clinical trials employing another strain of *Pediococcus spp.* revealed that the administration of *P. parvulus* decreased serum cholesterol levels and increased counts of faecal *Bifidobacterium spp.* (Mårtensson *et al.*, 2005).

Another group of LAB promoted as probiotics are enterococci, which reportedly help in the maintenance of normal intestinal microflora and stimulate the immune system (Bhardwaj *et al.*, 2008). Studies of potential probiotic properties of *E. faecium* showed its efficacy in reducing the recovery period of acute diarrhoea (Benyacoub *et al.*, 2003). Another study by Pieniz *et al.* (2014) showed that *E. durans* possessed antimicrobial activity and antioxidant ability and was resistant to simulated gastric juice and bile salts. Though enterococci have probiotic potential, they are considered opportunistic pathogens for humans as they might cause nosocomial infection and are also known to possess resistance to vancomycin (Tambyah, 2004). Additionally, Enterococci can cause haemorrhagic shock in pigs with possible subsequent transition of infection to humans (Lu *et al.*, 2002). Due to these controversial properties, the use of enterococci as probiotics remains under debate.

2.2.2 *Bifidobacteria*

Bifidobacteria are major constituents of the GIT microbiota of humans. They are Gram-positive, non-motile, often-branched, anaerobic saccharolytic bacteria (Gomes and Malcata, 1999). In the gut environment, bifidobacteria have a commensal relationship with their hosts, and contribute to host nutrition by utilizing complex carbohydrates, which are important sources of carbon and energy, but are not degraded in the stomach or intestine (Biavati, 1994). These substances include plant-derived dietary fibre and diet-related carbohydrates, such as starch, galactan, sucrose, amylopectin and pullulan (Ventura *et al.*, 2007, 2012). Their capacity to metabolize non-digestible dietary carbohydrates (prebiotics) can be used for selective stimulation of certain strains colonizing the intestinal tract. bifidobacteria used as probiotics include strains belonging to species of *Bifidobacterium lactis*, *B. bifidum*, *B. animalis*, *B. thermophilum*, *B. breve*, *B. longum*, *B. infantis* and *B. adolescentis* (Table 2.1). These bacteria have been shown to inhibit the adherence of enterotoxigenic *E. coli*, enteropathogenic *E. coli* and *C. difficile* to intestinal epithelial cells, an important trait for use of these bacteria as probiotics (Tsai *et al.*, 2008). Additional purported beneficial effects of bifidobacterial strains include the prevention or alleviation of infectious diarrhoea and the improvement of inflammatory bowel disease symptoms (Sanz, 2007). bifidobacteria have also been shown to modulate the host's immune response against other indigenous microflora (e.g., *B. adolescentis* down-regulates humoral immunity to *Bacteroides thetaiotaomicron*; Scharek *et al.*, 2000). Some bifidobacterial strains suppress *H. pylori*-induced genes in human epithelial cells (Shirasawa *et al.*, 2010) while other *Bifidobacterium spp.* cells and culture supernatants exerted inhibitory effects against *Streptococcus mutans* and *Streptococcus sobrinus*, important etiological agents in human dental caries (Lee *et al.*, 2011).

2.2.3 *Yeasts*

Saccharomyces boulardii is one of the best-studied probiotic species with a long history of use in treatment of multiple gastrointestinal disorders. The administration of this probiotic in lyophilized form was found effective in cases of diarrhoea by decreasing the duration of the disease, regardless of its cause (McFarland, 2007; Dinleyici *et al.*, 2012; Shan *et al.*, 2013). It has also been reported that *S. boulardii* prevented and treated relapses of inflammatory bowel disease, including moderate cases of ulcerative colitis (Guslandi *et al.*, 2000, 2003; Choi *et al.*, 2011). Interesting results have also been reported by Lim *et al.* (2015), suggesting that yeasts can enhance the growth

of other probiotics under acidic conditions: *Saccharomyces cerevisiae* EC-1118 was found to significantly enhance the viability of the probiotic strain *Lactobacillus rhamnosus* HN001 at pH 2.5 to 4.0. The use of *S. boulardii* in reduction of *C. difficile* infection *relapse* is still under debate due to controversial results of clinical trials (Flatley *et al.*, 2015). Among other yeasts species, *Torulaspora delbrueckii*, *Debaromyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces lactis*, *Kluyveromyces marxianus* and *Kluyveromyces lodderae* have shown strong antagonistic effect against pathogenic bacteria and high acid tolerance (Kumura *et al.*, 2004; Psani and Kotzekidou, 2006; Chen *et al.*, 2010). Despite an excellent record of safe use, yeasts may still be the cause of localized infections in immunocompromised patients (Thygesen, Glerup and Tarp, 2012).

2.2.4 *Akkermansia muciniphila*

Another recently-described microorganism with possible probiotic potential is *Akkermansia muciniphila* - a mucin-degrading bacterium that resides within intestinal mucus layers (Derrien *et al.*, 2004). According to several studies, obese patients have significantly lower amounts of this bacterium in their GIT (Collado *et al.*, 2008; Karlsson *et al.*, 2012). The genome sequence of *A. muciniphila* suggests the ability of this bacterium to metabolize a variety of complex carbohydrates, as well as synthesize multiple amino acids, vitamins and cofactors (van Passel *et al.*, 2011). Its influence on metabolic processes in the GIT has not been fully investigated; however, it has already been shown that this bacterium may be a potential treatment for obesity and its associated metabolic disorders (Everard *et al.*, 2013). Shin and colleagues have shown that oral administration of *A. muciniphila* to mice induced Foxp3 regulatory T cells in the visceral adipose tissue, which attenuated adipose tissue inflammation (Shin *et al.*, 2014). Based on these results it has been suggested that pharmacological manipulation of the gut microbiota in favour of *A. muciniphila* might be beneficial in the treatment of diabetes.

2.2.5 *Faecalibacterium prausnitzii* and other clostridia

Another bacterium that has been demonstrated to have a considerable impact on human gastrointestinal microbiota is *Faecalibacterium prausnitzii* of the *Clostridium* spp. cluster IV. This microorganism accounts for 5-15 % of the total fecal microbiota, making it one of the most abundant butyrate-producing bacteria in the GIT (Hold *et al.*, 2003; Flint *et al.*, 2012). Since butyrate is a primary energy source for intestinal epithelial cells, it is essential for maintenance of epithelial

barrier integrity. Based on animal studies multiple beneficial effects of butyrate also include reduction of cancer progression, protection against pathogens and stimulation of the immune system (Macfarlane and Macfarlane, 2011; Ríos-Covián *et al.*, 2016). The reduction of *F. prausnitzii* counts in fecal and biopsy samples has been observed in multiple studies of inflammatory bowel disease (especially, ileal Crohn's disease and ulcerative colitis) (Wang *et al.*, 2007; Swidsinski *et al.*, 2008; Andoh *et al.*, 2012). The first gnotobiotic rodent model with *F. prausnitzii* showed that it could influence gut physiology by modifying goblet cells and mucin glycosylation, thereby affecting the quality and quantity of produced mucus (Wrzosek *et al.*, 2013).

Other bacteria within the class Clostridia might also find use as potential probiotics, since they are highly abundant in human GIT microbiota and may play an important role in metabolism and immune system function. Atarashi and colleagues have shown that a mixture of 17 strains of *Clostridium spp.*, belonging to clusters IV, XIV and XVIII, were able to suppress experimental colitis in mice through induction of interleukin-10-producing regulatory T cells (Atarashi *et al.*, 2013). A similar mechanism of colitis suppression via IL-10 production by induced macrophages was observed using strain *Clostridium butyricum* MIYAIRI 588 (Hayashi *et al.*, 2013). According to another recent study, when mixed with *B. infantis*, *C. butyricum* was effective in treatment of experimental antibiotic-associated diarrhea in mice, and the beneficial effect of the mixture was superior to single clostridial strains (Ling *et al.*, 2015). However, though clostridia have potential for use as probiotics, there is still not enough evidence to support their medical efficacy and safety for humans.

2.3 *In vitro* and *in vivo* systems used to study probiotic effects

Novel probiotic-based strategies for therapeutic and prophylactic use against multiple GIT diseases are gaining popularity worldwide. Their effectiveness has been predicted by numerous animal model studies. However, the initial step in confirming probiotic effects is the extensive characterization of a bacterial strain to be used as a probiotic, which is usually performed under *in vitro* conditions by studying bacterial acid resistance, bile resistance, carbon source utilization, and aggregative properties, or *ex vivo* for their ability to adhere to mammalian cells (Kotikalapudi *et al.*, 2010; Wood *et al.*, 2012). Similarly, probiotic delivery methods, such as lyophilization or encapsulation, are also tested for their *in vitro* protective potential under simulated gastric conditions (Klemmer *et al.*, 2011; Wood *et al.*, 2012; Khan *et al.*, 2013; Wang *et al.*, 2014). The most popular

materials used for encapsulation of bacteria are alginate, carrageenans and gums, since they are easy to process, resistant to low pH and freezing, and are generally recognized as safe (Gbassi and Vandamme, 2012). We have recently reported the efficient delivery of *B. adolescentis*, encapsulated for this purpose in an alginate-pea protein protective matrix, into the lower gut of rats (Varankovich *et al.*, 2015a).

Apart from basic systems consisting of synthetic gastric juice solutions (low pH, 37°C), more complex systems have been developed, such as SHIME (Simulator of the Human Intestinal Microbial Ecosystem), designed to simulate different parts of human gastrointestinal tract (Cook *et al.*, 2012). Probiotic strains and methods for their delivery, preselected *in vitro*, are subsequently tested in animal models.

Traditionally-used animal models include mice and rats. Larger animals like rabbits, dogs and pigs are generally considered to have more common features with the physiology and microflora of the human gastrointestinal tract (Kararli, 1995). However, rodents are cheap, standardized, and have short life-cycles; thus, they have seen extensive use in large-scale research. Investigation of probiotic effects on animal microflora may be approached by: i) examining the quantitative and qualitative characteristics of bacterial microflora in animals using cultivation and/or molecular biology techniques, such as real-time polymerase chain reaction (qPCR), next-generation sequencing (NGS), and fluorescence *in situ* hybridization (FISH), or ii) evaluating treatment efficiency indirectly by using it to cure an artificially-induced disease.

Distribution of specific species of microorganisms is still being studied in healthy humans and compared with those of patients with various gastrointestinal diseases. Perturbations of microbiota, even in case of alterations in numbers of a single species (i.e. *A. muciniphila*), might be a cause (and an indicator) of the development of disease (Karlsson *et al.*, 2012). In this case, probiotic treatment might be useful in restoring microbiota balance in the gut. An interesting example of quantitative/qualitative analysis of animal gut microbiota after probiotic administration can be found in the study by Wang *et al.* (2015): 454 pyrosequencing of fecal bacterial 16S rRNA genes in obese vs. lean mice showed that the probiotic strains shifted the overall structure of the gut microbiota of obese animals toward that of lean mice fed a normal diet, with significant changes observed in 83 operational taxonomic units. Due to complicated analyses required to understand specific mechanisms of disease development, as well as the mode of action of a certain probiotic microorganism, the use of disease models is generally more widespread.

2.3.1 Rodent models of GIT diseases

Generally, in order to establish a disease model, mice are infected with the pathogen or irritant either one time or continuously (Pawlowski *et al.*, 2010; Bhinder *et al.*, 2013). Subsequently, animals are treated with probiotics with concomitant monitoring of the disease symptoms and evaluation of changes in the gut microflora. Following this approach, Verdú *et al.* (2008) infected mice with *Helicobacter pylori* for 4-6 months to investigate the effect of probiotic therapy on upper gastrointestinal dysfunction induced by chronic *H. pylori* infection. The authors reported that delayed gastric emptying in mice normalized significantly faster with probiotic treatment, compared to control groups, where the dysfunction was observed during a 2 month period after pathogen administration was ceased. Mice and rats have also been used to evaluate the efficiency of probiotics for the treatment of *Salmonella* and *E. coli* O157:H7 infections (Asahara *et al.*, 2001, 2004), inflammatory bowel disease (Shiba *et al.*, 2003) and immune suppression (Lollo *et al.*, 2012). Asahara *et al.* (2001) showed that intestinal growth and subsequent extra-intestinal translocation of orally-infected *Salmonella typhimurium* in mice were inhibited during administration of probiotic *B. breve*. Later, the same group reported *B. breve* was also effective in protecting mice against Shiga toxic-producing *E. coli* O157:H7 (Asahara *et al.*, 2004). Extrapolation of results achieved in animal studies and *in vitro* experiments to humans remains a difficult challenge. Many factors, such as differences in physiology and microflora composition of gastrointestinal systems, must be considered before interpreting the outcome.

The majority of *in vivo* experiments investigating the effects of probiotics on pathogenic bacterial populations use gnotobiotic mice (usually with human microflora systems in their GIT) (Bernet-Camard *et al.*, 1997; Aiba *et al.*, 1998, Gill *et al.*, 2001; Pawlowski *et al.*, 2010). For instance, in a study by Shiba *et al.* (2003), probiotic *B. infantis* 1222 was found to significantly suppress the systemic antibody response raised by *Bacteroides vulgatus*, a representative pathogenic *Bacteroides spp.* species, in a gnotobiotic mice model of inflammatory bowel disease. The use of conventional mice as a model for investigating human diseases is more problematic due to significant differences in animal and human gut microflora. Nevertheless, it is possible to use murine-specific organisms as models for the study of human pathogens. For instance, Ge *et al.* (2001) used *Helicobacter hepaticus* infection as an animal model for examining the pathogenesis of gastrointestinal diseases in humans caused by *H. pylori*. More recently, Bhinder *et al.* (2013) described the *Citrobacter rodentium* mouse model for the study of pathogen and host contributions

during infectious colitis. *Citrobacter rodentium* is a murine-specific bacterial pathogen, closely-related to enteropathogenic and enterohaemorrhagic strains of *E. coli* (Borenstein *et al.*, 2008). Several *C. rodentium* infection studies involving mice models have shown probiotics to reduce the severity of symptoms and prevent death caused by the pathogenic agent (Chen *et al.*, 2005; Gareau *et al.*, 2010; Rodrigues *et al.*, 2012; Mackos *et al.*, 2013). Chen *et al.* (2005) successfully treated *C. rodentium*-induced murine colitis with probiotic *L. acidophilus*. Gareau *et al.* (2010) similarly reported that *L. rhamnosus*, combined with *L. helveticus*, were effective in prevention and treatment of the same disease state in mice. Later, another group showed that *L. reuteri* was able to attenuate the severity of murine colitis caused by *C. rodentium* (Mackos *et al.*, 2013). Further investigation of host-pathogen and probiotic-pathogen interactions will likely provide better insight into treatment of *C. rodentium* infection in mice, and possibly *E. coli* infections in humans. However, confirmation of probiotic benefits and possible side effects will ultimately require human trials.

2.3.2 Human clinical trials

Human studies generally take the form of randomized clinical trials involving participants with some type of intestinal disorder. After assessment of eligibility and recruitment, participants are given either probiotic treatment or a placebo as a control. Results of these experiments have provided enough evidence for considering probiotics effective treatment for multiple GIT-associated diseases, such as acute gastroenteritis (Huang *et al.*, 2002), irritable bowel syndrome (Nikfar *et al.*, 2002) and necrotizing enterocolitis (Alfaleh *et al.*, 2011). Some trials showing the efficacy of bacteria of interest in the treatment of specific gastrointestinal disorders are listed in Table 2.2. In one recent trial aimed to assess the efficiency of *S. cerevisiae* in treatment of irritable bowel syndrome, 179 adults diagnosed with this condition were randomized to receive once-daily 500 mg of *S. cerevisiae* or placebo for 8 weeks. Cardinal symptoms (abdominal pain/discomfort, bloating/distension, bowel movement difficulty) were recorded daily after a 2-week run-in period. The results showed that abdominal pain/discomfort scores were significantly reduced during probiotic intake (Pineton *et al.*, 2015). A major trial involving 362 participants was conducted by Whorwell and colleagues in order to study the effect of *B. infantis* on symptoms of irritable bowel syndrome: probiotic administration lead to improvements in the majority of symptoms by more than 20 % compared to placebo (Whorwell *et al.*, 2006). Another human clinical trial proved the efficacy of *Lactobacillus GG* in treatment of *H. pylori* infection: daily administration of the probiotic led to

significant reduction in disease symptoms (diarrhea, nausea and taste disturbances) (Armuzzi *et al.*, 2001).

Though the majority of animal studies and human trials have not been successful in providing sufficient proof to substantiate claimed beneficial properties of probiotics, data from multiple lines of research involving humans suggests that certain strains of probiotic bacteria suppress gastrointestinal pathogens by simple competition by prevailing in numbers, and by producing antibacterial factors (bacteriocins and small organic molecules, such as fatty acids). However, more details into the mechanisms of action of probiotics on gut microbiota are essential.

Table 2.2. Some of the major human trials of probiotics for the treatment of gastrointestinal diseases

| Probiotic strain | Disease | Number of participants | Reported outcome | Reference |
|-----------------------------------|--|------------------------|---|-----------------------------------|
| <i>Lactobacillus rhamnosus</i> GG | <i>Helicobacter pylori</i> infection | 60 | Significant reduction ($p = 0.04$) of diarrhea, nausea and taste disturbances in the treatment group. | Armuzzi <i>et al.</i> , 2001 |
| | Antibiotic-associated diarrhea in children | 188 | Significant reduction of the incidence of antibiotic-associated diarrhea in children treated with oral antibiotics for common childhood infections. | Vanderhoof <i>et al.</i> , 1999 |
| | | 167 | The treatment effect on the incidence of diarrhea (95 % confidence interval) was -11 % (-21 % - 0 %). | Arvola <i>et al.</i> , 1999 |
| <i>Bifidobacterium bifidum</i> | Irritable bowel syndrome | 122 | Overall responder rates (decrease in symptoms severity) were 57 % in the treatment group, but only 21 % in the placebo group ($P=0.0001$) | Guglielmetti <i>et al.</i> , 2011 |
| <i>Bifidobacterium infantis</i> | | 362 | The improvement in overall symptom assessment exceeded placebo by more than 20 % ($p < 0.02$) | Whorwell <i>et al.</i> , 2006 |
| <i>S. cerevisiae</i> | | 179 | The proportion of responders, reporting improvement in abdominal pain/discomfort, was significantly higher ($p = 0.04$) in the treated group than the placebo group (63 % vs 47 %, OR = 1.88, 95 %, CI: 0.99-3.57). | Pineton <i>et al.</i> , 2015 |
| VSL#3* | Pouchitis | 40 | Three patients (15 %) in the treatment group had relapses of the disease within the 9-month follow-up period, compared with 20 (100 %) in the placebo group ($p < 0.001$). | Gionchetti <i>et al.</i> , 2000 |

Table 2.2. Some of the major human trials of probiotics for the treatment of gastrointestinal diseases. Continued.

| Probiotic strain | Disease | Number of participants | Reported outcome | Reference |
|----------------------------------|---|------------------------|--|---------------------------------|
| | Pouchitis | 40 | Two of the 20 patients (10 %) in the treatment group had an episode of acute pouchitis compared with 8 of the 20 patients (40 %) treated with placebo (log-rank test, $z = 2.273$; $p < 0.05$). | Gionchetti <i>et al.</i> , 2003 |
| | | 34 | Treatment of patients with mild to moderate stages of disease, not responding to conventional therapy, with probiotic resulted in a combined induction of remission/response rate of 77 % with no adverse events. | Bibiloni <i>et al.</i> , 2005 |
| | Ulcerative colitis | 124 | The efficacy of probiotic was significant (recurrence rate 34.6 %, compared with 64.7 % on placebo; $p = 0.04$) in patients with recurrent CDD, but not in patients with initial CDD (recurrence rate 19.3 % compared with 24.2 % on placebo; $p = 0.86$). | McFarland <i>et al.</i> , 1994 |
| | <i>Saccharomyces boulardii</i> <i>Clostridium difficile</i> -associated diarrhea (CDD) | 168 | A significant decrease in recurrences of CDD was observed only in patients treated with high-dose vancomycin (2 g/day) and probiotic (16.7 %), compared with those who received high-dose vancomycin and placebo (50 %; $p = 0.05$). | Surawicz <i>et al.</i> , 2000 |
| | | 211 | The mean (\pm SD) duration of diarrhea was 1.69 days (0.6) in patients given probiotic, compared with 2.81 days (0.9) in those given placebo. | Buydens and Debeuckelaere, 1996 |
| <i>Enterococcus faecium</i> SF68 | Acute diarrhea | 123 | The probiotic was shown to be effective in reducing the incidence of antibiotic-associated diarrhoea in comparison with placebo (8.7 % compared with 27.2 %, respectively). | Wunderlich <i>et al.</i> , 1989 |

Table 2.2. Some of the major human trials of probiotics for the treatment of gastrointestinal diseases. Continued.

| Probiotic strain | Disease | Number of participants | Reported outcome | Reference |
|--|---------------------------------------|------------------------|---|------------------------------|
| | Antibiotic-associated diarrhea | 94 | Prophylaxis with the probiotic significantly decreased the frequency of diarrhea from 71 % to 43 % (p = 0.019). | Black <i>et al.</i> , 1989 |
| Mixture of lactobacilli, bifidobacteria and streptococci | Travellers' diarrhea | 186 | Enteral administration of the probiotic in neonatal intensive care setup significantly reduced morbidity due to necrotising enterocolitis in very low birth weight newborn. | Samanta <i>et al.</i> , 2009 |
| Mixture of <i>B. infantis</i> , <i>B. bifidum</i> , <i>B. longum</i> and <i>L. acidophilus</i> | Necrotizing enterocolitis in newborns | | | |

*A mixture of *Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *Bifidobacterium longum*, *B. breve*, *B. infantis* and *Streptococcus salivarius* subsp. *Thermophiles*.

2.4 Immunomodulatory properties of probiotics

The high densities of microorganisms in the GIT can potentially have serious impact on the host immune system. Control of bacterial exposure to the immune system is accomplished by two mechanisms: i) stratification – minimization of direct contact between microflora and colon walls by mucus layers, and ii) compartmentalization – limiting bacterial exposure to specific intestinal sites with the help of antibacterial proteins (i.e., RegIII γ) and immunoglobulin A (IgA), which restrict bacterial numbers that contact epithelium (Hooper, 2012). Increase of IgA excretion provides better protection from pathogens; hence, probiotic strains capable of inducing excretion of this molecule could be beneficial for human health, since unlike many other antigens they are safe. Evidence for this was found in a study by Link-Amster *et al.* (1994) who proved that consumption of fermented milk containing *Bifidobacterium bifidum* and *Lactobacillus acidophilus* La1 following vaccination against *Salmonella typhi* Ty21 showed a significant increase in IgA serum concentration.

Another mechanism of immune effects of probiotics is their ability to influence monocyte-derived dendritic cells (DC) to drive the development of regulatory T-cells (T_{reg}). T_{reg} cells help to maintain tolerance to self-antigens. They limit the activity of T-helper cells, thus restricting the immune response, which is especially important during transplantation of organs and autoimmune diseases. Several studies have shown the ability of probiotic strains of *Lactobacillus reuteri*, *Lactobacillus casei* and *Bifidobacterium bifidum* to promote (through dendritic cells) the differentiation of T-cells into T_{reg} cells (Smits *et al.*, 2005; Lopez *et al.*, 2011; Lopez *et al.*, 2012).

Some probiotics can modulate the expression of different T-helper subsets. Alteration of the T_h1/T_h2 ratio towards T_h1 causes a pro-inflammatory effect, whereas increased excretion of T_h2 cells promotes an anti-inflammatory effect. In experiments on murine models it was found that *L. casei*, *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* increased the systemic production of IgG1, typical of a T_h1 response, and *L. acidophilus* enhanced production of IgG2a (T_h2 response) (Perdigon *et al.*, 2002).

Results of many studies involving different probiotic strains suggest the possibility of specific application of various probiotic strains depending on the kind of systemic reaction that is needed (Perdigon *et al.*, 2002; Mohamadzadeh *et al.*, 2005; Rigby *et al.*, 2005). However, no pattern has been discovered even at the species-level, which means that each bacterial strain has to be examined separately for its effect on immune system.

2.5 Characterization of complex microbial communities

2.5.1 Culture-dependent and culture-independent methods

Complementary information received from both culture-dependent and culture-independent techniques is generally required to generate a comprehensive view of a microbial community.

Traditional culture-dependent methods include using selective media for detection and cultivation of specific genera of microorganisms. This approach is well-suited for quantifying bacterial numbers, does not require special equipment, and is able to provide physiological and morphological data. However, not every microbe is culturable and not every metabolic and physiological requirement is reproduced *in vitro* (Spratt, 2004). Due to these limitations, selective media typically allows the detection of a relatively-small fraction of microorganisms; hence, cultural analyses may produce erroneous or biased results. In addition, cultivation and subsequent identification of isolates using culture-dependent techniques is also time-consuming.

The advent of molecular-based techniques has greatly improved the precision and reliability of research outcomes and allows the analysis of non-cultivable members of the bacterial community (Tannock, 2002). Classical culture-independent methods include the polymerase chain reaction (PCR; specific amplification of a target DNA sequence), fluorescence *in situ* hybridization (FISH; direct detection of a target DNA or RNA site by a fluorescently-labelled probe molecule), denaturing gradient gel electrophoresis (DGGE), where DNA with the identical sequences migrate within a gel the same distance forming a “band” (Ye *et al.*, 2001; Namsolleck *et al.*, 2004; Sha *et al.*, 2013). The DGGE analysis of the PCR-amplified 16S rRNA gene is now routinely-used to examine the diversity of microbial communities. The improved version of this reaction – real time PCR (qPCR, RT-PCR), allows amplifying and simultaneously-quantifying a targeted DNA molecule; hence, it has become a widely-used tool for analyzing microbiological communities (Heid *et al.*, 1996; Gibson *et al.*, 1996; Kanno *et al.*, 2009). Though new technologies are constantly being developed and tend to be more effective in terms of sequence coverage, the ability of PCR to specifically-target particular markers, even at the strain level, allow it to remain a popular method in the molecular microbiology (Smith and Osborn, 2009).

2.5.2 Next-generation sequencing and metagenomics

Over the last two decades, capillary-based, semi-automated sequencing technology has gradually been replaced by next-generation sequencing (NGS) (Swerdlow and Gesteland, 1990;

Shendure and Ji, 2008; Karger and Guttman, 2009; Scholz *et al.*, 2012). Several platforms used in NGS involve different biochemistry approaches; however, they have common functional scheme which includes the following steps: 1) fragmentation of DNA, 2) addition of adaptors/linkers; 3) purification, 3) amplification (on beads or glass), and 4) sequencing (by DNA-polymerase synthesis or ligation). Amplification steps as well as sequencing techniques differ for every platform. The 454 Sequencing (Life Sciences, 2004) and SOLiD (Life Technologies, 2008) platforms use emulsion-PCR, where the PCR reaction takes place in oil-in-water emulsion. Illumina technology involves bridge-PCR on the surface of glass flow cells (Fedurco, 2006; Mardis, 2008; Turcatti, 2008). The Ion Semiconductor Sequencing (or Ion Torrent), released by Ion Torrent Systems Inc. in 2010 (Rusk, 2011) uses detection of hydrogen ions that are released during the polymerization of DNA, which is promoted as being a more-sensitive approach for the detection of each sequencing step than the use of CCD-cameras and lasers for detecting of light flashes (454 Sequencing) or fluorescence (Illumina) (Merriman *et al.*, 2012).

Perhaps the most promising area for high throughput DNA sequencing is the nanopore sequencing, which allows identification of single molecules using nanopores, built into a synthetic membrane (Clarke *et al.*, 2009). Minute changes in ionic current across a membrane are measured and recorded when a single DNA molecule is driven through a nanopore (Stoddart *et al.*, 2009). In contrast to other sequencing techniques, this approach allows direct targeting of single DNA molecules, enabling real-time sequencing, where reads are available for analysis as soon as they have passed through the sequencer. Though the platform has a high error rates (~ 12-35 % in reading accuracy) its real-time nature might be particularly important for clinical diagnostic applications and pathogen detection (Ashton *et al.*, 2015; Laver *et al.*, 2015; Chu *et al.*, 2017).

Both classical molecular biology methods and next-generation sequencing are widely-used for examining environmentally-derived samples of microbiological communities, as well as for animal and human microflora analysis. The genomic analysis by direct extraction and analysis of DNA from an assemblage of microorganisms is referred to as metagenomics and allows by-passing the need to isolate and culture individual members of a complex community, using direct genomic analysis instead (Handelsman, 2004). Generally, metagenomic analysis includes isolating of DNA, amplifying all genes of interest found in the sample, sequencing PCR products and, finally, classifying the data according to a database of assigned sequences. The gene of interest for analysis of bacterial communities is most commonly 16S rRNA; however chaperonin-60 (*cpn60*) gene was

also used for this purpose (Links *et al.*, 2012). Examples of the metagenomic approach for the analysis of microflora can be found in the works of Ventura *et al.* (2009) and Riboulet-Bisson *et al.* (2012). The alternative to amplicon metagenomic sequencing is a shotgun approach, where all DNA, extracted from the community, is sheared into tiny fragments that are independently sequenced (Sharpton, 2014).

Successful metagenomic analysis of a microbial community might be limited by a shortage of reference genomes. This limitation will seemingly be resolved soon with the rapid development of relevant and specialized databases. One of the major global research initiatives that has produced a massive amount of genomic data is The Human Microbiome Project (HMP). It was launched in 2008 by an initiative of United States' National Institutes of Health and should be finished by the end of this year. HMP “focuses on describing the diversity of microbial species that are associated with health and disease” (Nelson *et al.*, 2010). Different laboratories working on the project are using culture-independent approaches for microbial community characterization, such as metagenomics or whole genome sequencing. Detailed information and extensive databases can be obtained at www.hmpdacc.org.

2.6 Prebiotics

According to Roberfroid (2007), a prebiotic is a “selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confers benefits upon host well-being and health”. Currently, the majority of prebiotics used in food manufacturing are short-chain carbohydrates consisting of 3-10 monomers that are non-digestible by human or animal enzymes (Saad *et al.*, 2013). There are a large variety of these substances, including: inulin, fructo-oligosaccharides (FOS), galactooligosaccharides (GOS), isomalto-oligosaccharides (IMO), xylo-oligosaccharides (XOS) and soy oligosaccharides (SOS). Most of these compounds are synthesized from more fundamental molecules (i.e., sucrose) or isolated from the depolymerisation of plant polysaccharides (Ouwehand, 2007; Kelly, 2009). In food production, prebiotics see application as either as food supplements (powders, syrups) or are incorporated into food products (yogurts, breads) (Charalampopoulos and Rastall, 2012).

Claims associated with prebiotic use include optimized colonic function and metabolism, reduced colonic pH, decreased nitrogenous end-products and reductive enzyme production, increased expression of binding proteins or certain biomarkers in the field of lipid and mineral

metabolism, and immune system modulation (Roberfroid, 2007; Saad *et al.*, 2013). When combined with probiotic microorganisms (*Lactobacillus spp.*, *Bifidobacterium spp.*), prebiotics are hypothesized to increase populations of helpful commensal bacteria (Davis *et al.*, 2010), inhibit various human and animal pathogenic bacteria strains (Fooks *et al.*, 1999; Saad *et al.*, 2013), contribute to the treating of *Clostridium difficile*-associated diarrhoea and colitis disorders (Licht *et al.*, 2012), and modify immune response by stimulating or inhibiting the secretion of various cytokines and interferons, modulating of NK (natural killer) cell activity and effecting lymphocyte proliferation (Delgado *et al.*, 2011).

Inulin-type prebiotics are fructans that have $\beta(2\rightarrow1)$ fructosyl-fructose glycosidic bonds and are resistant to enzymatic hydrolysis in the human GIT (Kelly, 2009). This class of prebiotics includes inulins and fructooligosaccharides (FOS). Inulins are polysaccharides, composed of 20 to several thousand fructose units, joined by a $\beta(2\rightarrow1)$ glycosidic bonds, with a terminal glucose. Fructooligosaccharides (FOS) are a subgroup of inulins, consisting of polymers with a degree of polymerization (DP) ≤ 10 . Several clinical trials demonstrated a significant bifidogenic ability of inulin and its short-chain derivatives (Gibson *et al.*, 1995; Buddington *et al.*, 1996; Kleessen *et al.*, 1997; Closa-Monasterolo *et al.*, 2013). It has also been reported that the addition of FOS to microcapsules containing *B. adolescentis* improved survival of the bacterium under simulated stomach conditions (Wood, 2010).

One more prospective group of prebiotics are xylo-oligosaccharides (XOS) – sugar oligomers made up of xylose units linked through $\beta(1\rightarrow4)$ -bonds (Aachary *et al.*, 2011). According to Zeng *et al.* (2007), XOS accelerated the growth of *B. adolescentis in vitro* by 20 %. Investigation of fermentability of XOS by probiotic bacteria showed higher growth rates for *B. adolescentis* than *B. longum*, *B. infantis* and *B. breve* (total XOS consumption from the medium reached 77 % after 24 h) (Gullon *et al.*, 2008). Arabinoxylan oligosaccharides (AXOS), which are XOS with arabinose side chains, are new prebiotics with the already-proven ability to increase the concentration of bifidobacteria in the intestines of animals (Van Craeyveld *et al.*, 2008; Neyrinck *et al.*, 2012). Results of clinical trials have also revealed the significant increase in the population of bifidobacteria after AXOS intake, thus suggesting the use of these polysaccharides as prospective prebiotics (Cloetens *et al.*, 2008).

2.7 Protective methods and coatings

The effective delivery of probiotics in sufficiently high numbers to the intestine for a benefit to be realized remains a practical challenge. When entering the stomach, bacteria are exposed to adverse environmental conditions such as low pH and pepsin. The probiotic food product should contain protective substances that prevent the number of viable bacteria from becoming reduced so that they can reach the colon in sufficient number in order to provide a functional effect or benefit to the human or animal host. The most efficient method for protection of bacteria from harmful influence of acid and bile is their encapsulation within single or multiple biopolymer coatings, varying of biopolymer concentration and increasing of initial cell loads (Chandramouli *et al.*, 2004; Petrović *et al.*, 2007; Annan *et al.*, 2008). The survival of bacteria is related to the structural integrity of capsules, which involves various testing of different wall components to select favourable variants, such as microcapsules that are insoluble at acidic pH but dissolve when exposed to the alkaline pH of the intestine (Narayani and Rao, 1995a, 1995b; Nickerson *et al.*, 2006). Capsules also need to be non-toxic and pepsin-resistant.

One additional important factor that has a significant impact on the survival of bacterial cells in acidic environments is the size of the capsules. To ensure significant protection, microcapsules should be 1-3 mm in size, a size that can be unfavourable for the incorporation of microcapsules into food products due to textural/organoleptic effects (Champagne and Fustier, 2007). Alternatively, microcapsules with diameters 40-80 µm were insufficient to provide the required protection (Truelstrup Hansen *et al.*, 2002). In any event, the size of the protective microcapsules need to be determined in each case according to the intended use.

With respect to capsule wall composition, preference is usually given to natural hydrocolloids and gums that are generally recognized as safe (GRAS) for human health and show good protection of bacterial cells when challenged with the acidic environment of the gut: alginate, carrageenans and gums (Holzapfel and Schillinger, 2002; Shah *et al.*, 2007; Bajaj *et al.*, 2007; Morris *et al.*, 2012). These biopolymers possess such useful features as viscosity (thickening or gelling), water binding, emulsion stabilization, adhesion, foam stabilization and film formation (Chaplin, 2012). Due to these properties, it is easy to achieve various textures and forms by altering and combining physical factors.

Two commonly-used polymers for immobilizing viable bacterial cells are sodium alginate and *k*-carrageenan (McMaster *et al.*, 2005; Petrović *et al.*, 2007; Annan *et al.*, 2008). Gellan and

xanthan have also been used for capsule formation. These two polymers, when applied as a mixture, show significant protection of cells from synthetic stomach juice *in vitro* (Sun and Griffiths, 2000; McMaster *et al.*, 2005). Another microencapsulation variation involves rennet-gelation of milk proteins and compression capsulation (bacterial powders are first compressed into a pellet, which is then encapsulated with a coating material of a combination of sodium alginate and hydroxypropyl cellulose by further compression) (Chan and Zhang, 2005; Heidebach *et al.*, 2009).

One of the main criteria for success of any candidate protective coating approach is for it to promote the survival of bacteria in amounts equal to or greater than $7 \text{ Log CFU/ml}^{-1}$ after being exposed to unfavorable conditions in stomach (Kotikalapudi *et al.*, 2009).

2.7.1 Alginate

According to many studies examining the immobilization of live bacteria (Champagne and Fustier, 2007; Annan *et al.*, 2008; Chávarri *et al.*, 2010; Voo *et al.*, 2011), the most common material for encapsulation is alginate. This biopolymer is of algal origin and composed of (1 → 4)-linked β -D-mannuronic (M) and α -L-guluronic acid (G) residues (Voo *et al.*, 2011). Water-holding capacity and solubility of alginate depends on various extrinsic factors such as pH, ionic strength and the origin of ions. For example, when sodium alginate is added to aqueous calcium chloride, it forms a water-insoluble, gelatinous substance known as calcium alginate (Fig. 2.1). This property of alginate has been widely-used for the entrapment of probiotic bacteria. Bacterial cultures are added into an aqueous alginate solution, and in the presence of calcium chloride, leads to the formation of alginate microcapsules containing the bacteria.

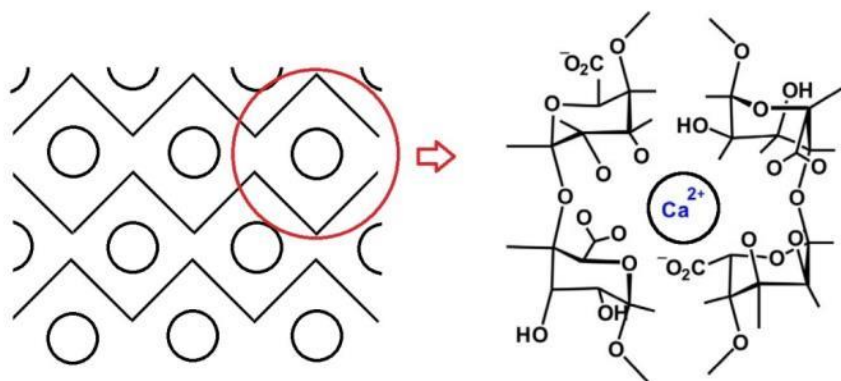


Figure 2.1. Two-dimensional network structure of calcium alginate (adapted from Degen *et al.*, 2015).

Since *Bifidobacterium* strains prefer anaerobic conditions, compared to other probiotic microorganisms (e.g., streptococci), it is preferable to use alginate microcapsules for their delivery into the GIT (Champagne and Fustier, 2007). Though alginate is widely-used for bacterial cell encapsulation, it is associated with certain disadvantages, including low tolerance to stomach acids (Krasaekoopt *et al.*, 2003).

2.7.2 Iota-carrageenan

Carrageenans are also extracted from algae, specifically from *Eucheuma spinosum* (Gunning *et al.*, 1998). These algal polysaccharides have a linear structure and high molecular weight. They are composed of sulfated and non-sulfated galactose and 3,6-anhydrogalactose units, joined by alternating -(1,3) and -(1,4) glycosidic bonds. Various types of carrageenans (*kappa*, *iota*, *lambda*) differ mostly in their degree of sulfate substitution (Hambleton *et al.*, 2009). *Iota* carrageenan has more sulphate groups than *kappa*, which makes it more hydrophilic (Luna *et al.*, 2010; Fig. 2.2).

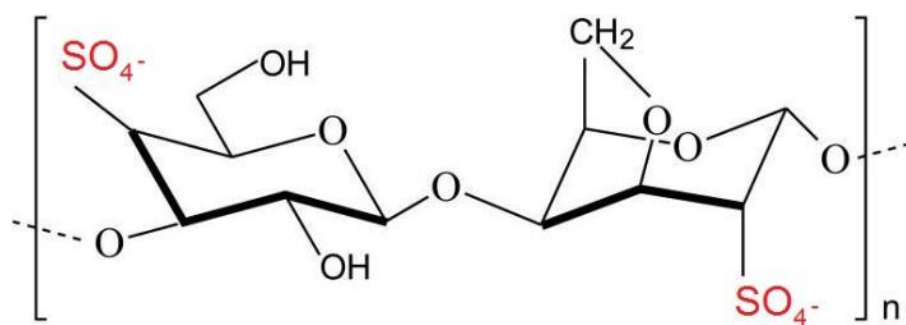


Figure 2.2. Molecular structure of *iota* carrageenan (adapted from Hamman, 2010).

In aqueous solutions, *iota*-carrageenan dissolves at temperatures 60 to 80°C and produce thermo-reversible gels on cooling to room temperature (Gbassi and Vandamme, 2012). However, high temperatures combined with pH < 3.5 can cause damage to the molecular structure. In comparison with *kappa*-carrageenan, the *iota*- form is more stable after being subjected to freeze-drying (Imeson, 2000). According to an X-ray investigation conducted by Janaswamy and Chandrasekaran (2002), calcium ions and water molecules mediate interactions between the sulfate groups of neighboring helices, thereby stabilizing the three-dimensional structure of *iota*-carrageenan.

2.7.3 Gellan gum

Gellan gum is produced by the aerobic, Gram-negative bacterium, *Pseudomonas elodea*, as a fermentation product which is extractable with isopropyl alcohol. It is an anionic deacetylated exocellular polysaccharide of high molecular weight (Shah *et al.*, 2007). Gellan gum structure is based upon repeating tetrasaccharide units that include one α -L-rhamnose, one β -D-glucuronic acid and two β -D-glucose residues (Fig. 2.3).

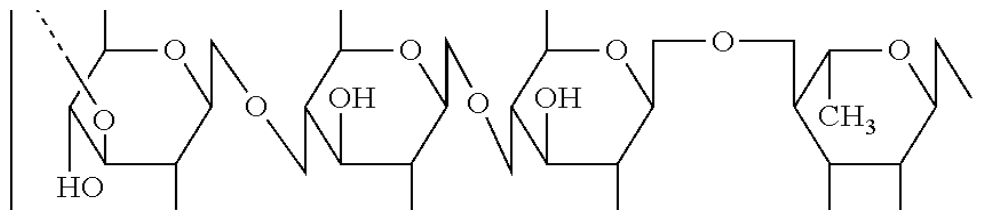


Figure. 2.3. Repeating units of chemical structure of deacetylated gellan gum (adapted from Bajaj, 2007).

Deacylation transforms soft, elastic thermo-reversible native gellan gum to harder and more brittle gels with higher thermal stability. Deacylated gellan gums are water-soluble, resistant to pH 3.5-10 and form double-helices on cooling in the presence of monovalent or divalent cations, which results in gelation (Shah *et al.*, 2007; Morris *et al.*, 2012). Several studies have demonstrated the utility of using gellan gum as a wall material for probiotic-containing microcapsules. However, others have reported that it is better to use gellan gum in combination with other polymers, such as xanthan gum, jamican and sodium alginate, as it changes viscoelastic behaviour of the mixture, improving functional properties of capsules (Chen *et al.*, 2007; Jiménez-Pranteda *et al.*, 2012a, 2012b).

2.7.4 Pea protein isolate

The use of pea protein isolate (PPI) in the food industry is gaining popularity due to its hypoallergenic properties and low concentrations of by-products (8-12 % (v/w) of the protein) (Franco *et al.*, 2000; Tömösközi *et al.*, 2001). PPI is extracted from field peas (*Pisum sativum*) and contains two major globulin proteins: legumin (Lg; 350-400 kDa) and vicilin (Vn; 150 kDa) (Ducel *et al.*, 2004). Several studies have proven the advantages of using PPI in combination with calcium alginate during the formation of probiotic-containing microcapsules (Kotikalapudi *et al.*, 2010; Klemmer *et al.*, 2011), where PPI enhanced protection of bacterial cells in the simulated gastric

juice. In order to form stable interactions with polyanions such as alginate, carrageenan or gellan gum, PPI requires an overall positive charge. Since the isoelectric point of PPI is $\text{pH} \sim 4.5$, favourable conditions for coacervation would occur at $\text{pH} < 4.5$. At $\text{pH} > \text{pI}$, PPI is negatively-charged, which leads to segregative phase separation upon mixing with polyanions. In order to determine the proper conditions for formulation of stable interactions between protein and polysaccharide, the influence of various factors on complex formation needs to be evaluated (e.g., concentration, ratio, biopolymer-type, reactive groups present, molecular weight, pH, temperature, concentration of ions in the solution) (de Kruif *et al.*, 2004).

3 IN VITRO AND IN VIVO EVALUATION OF PROTEIN–POLYSACCHARIDE MATRICES FOR ENCAPSULATION OF ACID-SENSITIVE BACTERIA²

3.1 Abstract

Protein-polysaccharide microcapsules containing *Bifidobacterium adolescentis* were produced and tested in a series of *in vitro* survival experiments to evaluate capsule protection of the bacterium to simulated stomach conditions, as well as their ability to release the encapsulated bacteria under conditions similar to those found in the lower gut. A protein fraction isolated from peas (pea protein isolate: PPI; 2.0 %; w/v) was mixed with each of three different polysaccharides (0.5 % (w/v) of either sodium alginate, *iota*-carrageenan and gellan gum) to produce microcapsules ranging in size from 2 to 3 mm diameter. All capsule formulations provided significant protection for cells exposed to synthetic stomach juice at 37°C relative to non-encapsulated bacteria. In addition, PPI-alginate and PPI-*iota*-carrageenan microcapsules were found to dissolve in simulated intestinal fluid at 37°C, releasing 70-79 % of their bacterial “payload” within 3 h, with higher cell numbers being released from the freeze-dried capsules. PPI-gellan gum microcapsules did not dissolve to the same extent and the number of released cells was ~ 26-30 % lower. Following a temporal rat feeding study with the test bacterium encapsulated in PPI-alginate, *B. adolescentis*-specific PCR and qPCR analyses confirmed the presence of DNA from this species in rat feces, but only during the period of capsule intake.

3.2 Introduction

The term probiotic refers to living microorganisms that confer a health benefit, when administered in adequate amounts (FAO/WHO, 2001). The most common microorganisms claimed to function as probiotics are *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, yeasts and bacilli strains.

²Varankovich, N. V., Khan, N. H., Nickerson, M. T., Kalmokoff, M., and Korber, D. R. (2015a). Evaluation of pea protein–polysaccharide matrices for encapsulation of acid-sensitive bacteria. *Food Res Int* 70, 118–124

In 2008, the global probiotic supplement market (i.e., pills, caplets) was worth approximately \$1.5 billion, and is expected to reach \$31.1 billion by 2015, with a compound annual growth rate of 7.6 % over the previous 5-year period (Champagne *et al.*, 2011; Pedretti, 2013).

The delivery of beneficial bacteria to the gastrointestinal system of the host is often implemented in two different ways: either utilizing conventional pharmaceutical delivery systems (polysaccharide or protein capsules, beads, etc.) or through direct incorporation in commercial products, such as yogurts, creams or milk (Govender *et al.*, 2014). While the latter type is rarely regulated or controlled in terms of probiotic bacterial loads, conventional systems differ greatly in formulation processes and utilize materials of various origins: seaweed (carrageenan, alginate), other plants (starch, gum Arabic), bacteria (gellan and xanthan gums) and animal proteins (milk, gelatin) (Rokka and Rantamäki, 2010). Over the past decade, plant-based proteins have been experiencing greater market growth than seen for proteins of animal origin, such as in the case of widely-used gelatin, casein, ovalbumin and whey (Klassen, 2010). Currently, the most promising approach for the use of plant proteins in the area of probiotics is to blend them with polysaccharides to improve protein functionality and create protective capsules containing immobilized probiotic bacteria (Klemmer *et al.*, 2011; Jiménez-Pranteda *et al.*, 2013; Khan *et al.*, 2013). The ideal capsule would allow the protection of entrapped cells during exposure to the harsh conditions of the upper gastrointestinal tract, followed by the release of bacteria in the lower gut. Encapsulation within single or multiple biopolymer coatings was proposed as an effective method to protect potential probiotics from the harmful effects of stomach acid and bile (Sun and Griffiths, 2000; Chandramouli *et al.*, 2004; Petrović *et al.*, 2007; Annan *et al.*, 2008). With respect to capsule wall composition, preference has usually been given to natural hydrocolloids and gums that are generally recognized as safe (GRAS) and which show good protection of bacterial cells against acidic environment of the stomach; for example, alginate, carrageenan and gums (Holzapfel and Schillinger, 2002; Shah *et al.*, 2007; Bajaj *et al.*, 2007; Morris *et al.*, 2012). Sodium alginate, *iota*-carrageenan and gellan gum are widely-used by the food industry and undergo gelation in the presence of divalent calcium ions, making them ideally suited for the extrusion-based encapsulation process. However, with the exception of alginate, their use as encapsulating agents has been limited. Capsules containing probiotics are also often dried for distribution and storage. The most promising technology in this regard is freeze-drying, due to milder conditions of this process that helps to prevent excessive losses

of cell viability (Tsen *et al.*, 2002; Kurtmann *et al.*, 2009; Semyonov *et al.*, 2010; Martin-Dejardin *et al.*, 2013).

The goal of this study was to further examine the suitability of pea protein isolate mixed with sodium alginate, *iota*-carrageenan or gellan gum, as protective materials for acid-sensitive *Bifidobacterium adolescentis* under simulated stomach conditions, along with the ability of the microcapsules to release bacterial cells under simulated conditions of the lower intestinal tract using both fresh and freeze-dried capsules. The efficacy of pea protein isolate-alginate (PPI-alginate) microcapsules as a delivery system for *B. adolescentis* was also tested *in vivo* using an animal model system.

3.2 Materials and methods

3.2.1 Ethics statement

The study was carried out in strict accordance with the Canadian Council on Animal Care Guidelines. Ethical approval for the animal study was obtained from Health Canada (Ottawa, Canada) (Protocol No. 2013-008).

3.2.2 Materials

Pea protein isolate was kindly donated by Nutri-Pea Ltd. (Portage La Prairie, MB, Canada). Alginic acid sodium salt, calcium chloride dihydrate, Tween 80 and *iota*-carrageenan were purchased from Sigma-Aldrich (Oakville, ON, Canada). Deacetylated gellan gum (Kelcogel F) was purchased from CP Kelco U.S., Inc. (San Diego, CA, USA).

3.2.3 Bacteria and culture conditions

Bifidobacterium adolescentis ATCC[®] 15703[™] was stored at -80°C in a 1:2 (v/v) suspension of glycerol and MRS broth. Prior to bacterial encapsulation, frozen stock was plated on de Man, Rogosa and Sharpe (MRS; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) semi-solid medium containing 0.05 % (v/v) L-cysteine and 1.5 % (w/v) agar and then incubated for 24 h at 37°C under anaerobic conditions (80 % N_2 , 10 % CO_2 and 10 % H_2) in an anaerobic chamber (Forma Scientific Inc., Marietta, GA, USA). Pure colonies of *B. adolescentis* were then transferred from plates to 50 mL tubes containing 45 mL of MRS-cys broth for incubation under the same conditions for 24 h, which corresponded to the stationary phase of growth (Wood, 2010). Bacterial cells were then

recovered by centrifugation (2000 g x 12 min) (Sorvall SS-1, Sorvall Inc., Newtown, Connecticut, USA), washed twice with 1.0 % alkaline peptone water (APW; 10 % (w/v) peptone, 10 % (w/v) sodium chloride; pH 8.5) and used for preparation of capsules.

3.2.4 Preparation of bacteria-containing capsules

The methodological approach of this research was based on encapsulation and extrusion techniques used by Klemmer *et al.* (2011) and Krasaekoopt *et al.* (2003), with modifications. The composition of the wall material was as follows: 2.0 % PPI + 0.5 % (w/w) polysaccharide (alginate, *iota*-carrageenan or gellan gum). Concentration of PPI was adjusted to the protein content of the isolate (85 %); whereas, the polysaccharide concentration was used as is, on a per-weight basis. Protein isolate was dissolved at 85°C and pH 9.0 for 2 h. Then pH was then adjusted to 7.0 and a specific polysaccharide was added to the mixture. After complete dissolution of the polysaccharide (1-1.5 h at 85°C), the solution was cooled to room temperature (21-23°C) and mixed with a 2.0 % (v/v) bacterial slurry preparation, recovered by centrifugation from 24 h bacterial broth culture (optical density at 600 nm was equal to 1.305 (n=3) as measured using a Genesys 10S UV-Vis Spectrophotometer, Thermo Fisher Scientific, Madison, WI, USA).

The final mixture (a blend of protein, polysaccharide and bacteria) was then extruded using a syringe and 18G (G, gauge) needle into 200 mL of deionized distilled water (ddH₂O) containing Ca²⁺ ions (cross-linking agent) and Tween 80 (emulsifier). The concentrations of Ca²⁺ (CaCl₂) and Tween 80 were determined empirically, depending on their effect on uniformity of capsule morphology and protective properties of capsules. For PPI-sodium alginate capsules, concentrations of CaCl₂ and Tween 80 were 1.0 % (w/v) each; for PPI-*iota*-carrageenan microcapsules they were 2.0 % (CaCl₂; w/v) and 1.5 % (Tween 80; w/v); and for PPI-gellan gum capsules, concentrations of 2.25 % (CaCl₂; w/v) and 1.0 % (Tween 80; w/v) were used. Lower concentrations of CaCl₂ were not sufficient for proper cross-linking to occur in order to form a capsule, whereas at higher CaCl₂ concentrations, irregular-shaped microcapsules (i.e., bell-shaped) were formed. Prepared microcapsules were immediately used in subsequent survival and release experiments, as described below (and labeled as ‘fresh’ capsules). To investigate the performance of dried microcapsules with immobilized *B. adolescentis*, microcapsules containing bacteria were freeze-dried (Labconco FreeZone, Kansas City, MO, USA) for 15 h and then were used in survival and release studies, conducted according to methods described below (and labeled as ‘freeze-dried’ capsules).

3.2.5 Survival of *B. adolescentis* in PPI-polysaccharide microcapsules under simulated stomach conditions

For survival studies, aliquots of 0.1 g of fresh or 0.01 g of freeze-dried microcapsules were added to centrifuge tubes (2 mL) containing 0.9 mL (or 0.99 mL in case of dried capsules) of synthetic stomach juice (SSJ; 8.3 g/L proteose peptone, 3.5 g/L glucose, 2.05 g/L NaCl, 0.6 g/L KH_2PO_4 , 0.11 g/L CaCl_2 , 0.37 g/L KCl, 0.05 g/L bile salts, 0.1 g/L lysozyme, 13.3 mg/L pepsin; pH 1.8) (Pedersen *et al.*, 2004; Khan *et al.*, 2013) and incubated for 2 h at 37°C. Starting at time 0, then every 5 min over the first 30 min, and subsequently every 30 min, tubes of each capsule type (fresh and freeze-dried, PPI-alginate, -*iota*-carrageenan and -gellan gum capsules) were removed from the incubator. Capsules were immediately washed with 1.0 % APW, homogenized (Omni-mixer, Sorvall Inc., Newtown, USA) at 2,000 rpm for 2 min, serially-diluted with 1.0 % APW and plated on MRS-cys agar to evaluate cell survival. The same procedure, but without SSJ treatment, was used to determine the initial counts of cell within capsules. Plates were incubated at 37°C in an anaerobic chamber for 48 h and then enumerated and reported as log CFU/g. Survival of non-encapsulated free bacterial cells were determined according to the same method, but without homogenizing, and was used as a control. Experiments were conducted in triplicate. The amount of time for a 1-log reduction in cell number to occur was determined and represented as the *D*-value, which was calculated by the end-point method, utilizing initial and terminal time points: $D = U/(\log N_0 - \log N_u)$, where *D* is the *D*-value, *U* = exposure time, N_0 = CFU/g at 0 min, N_u = CFU/g at 120 min.

3.2.6 Release of *B. adolescentis* from PPI-polysaccharide microcapsules under simulated intestinal conditions

For cell release studies, 0.1 g of fresh- and 0.01 g of freeze-dried microcapsules of each type were added to 9.9 mL or 9.99 mL of simulated stomach juice, respectively, and then incubated for 2 h at 37°C. After 2 h, 0.1 g of microcapsules were removed from SSJ and added into 9.9 mL of simulated intestinal fluid (1.25 % (w/v) NaHCO_3 , 0.60 % (w/v) bile salts, 0.09 % (w/v) pancreatin; pH 6.5) and incubated under anaerobic conditions for 3 h at 37°C. Every hour, a 0.1 mL aliquot of the sample was removed, serially-diluted using 1.0 % APW, and plated on MRS-cys agar. The plates were incubated at 37°C in anaerobic chamber for 48 h and then enumerated. Viable counts were conducted in triplicate.

3.2.7 Size of bacteria-containing capsules

PPI-polysaccharide-bacteria mixtures were extruded through an 18G needle into cross-linking solution. The size of fresh and freeze-dried microcapsules was measured using an electronic digital caliper (model 62379-531, Coltrol Company, Friendswood, TX, USA). Fifteen microcapsules from two separate batches for each capsule type were measured and averaged.

3.2.8 Confocal Laser Scanning Microscopy (CLSM)

The internal structure of bacteria-containing microcapsules was analyzed by CLSM. Both fresh and simulated stomach juice-treated microcapsules were cut in half with a sterile scalpel, stained and immediately immobilized on the surface of a cover glass using molten 0.8 % (w/v) agar, tempered to 40-45°C. After the agar solidified, the agar cover glass was inverted and transferred to a glass chamber made from glass slides and filled with tempered, but still liquid agar, for further immobilization of the capsules. Staining of the microcapsules was performed using SYTO 9 (5 mM solution in DMSO; Life Technologies™ Corp., Carlsbad, CA, USA) according to the guidelines provided by Life Technologies™ (SYTO 9 Manual, 2011). The dye (1 µL) was then added to 2 ml water, vortexed, directly applied to the capsule surface and then incubated for 15 min in the dark. Glass chambers with stained bacteria-containing microcapsules were analyzed by a Nikon C2 CLSM microscope (Nikon, Mississauga, ON, Canada) equipped with a 60X Plan Apo VC (N.A. 1.4, Nikon) objective lens and a blue diode 488 nm laser. Three representative locations on the samples were randomly-chosen for obtaining optical thin sections of the capsule surface and stained bacteria. Accordingly, a series of *xy* confocal images of microcapsules were obtained over the *z*-dimension (to a 50 µm depth) using a *z*-step increment of 0.5 µm (or 1.0 µm in case of fresh PPI-alginate capsules). Each image was taken with a *xy*-resolution of 512 by 512 pixels and a pinhole radius of 20. Selected stacks of images were then converted to maximum intensity projections.

3.2.9 Animal feeding study

PPI-alginate microcapsules containing *B. adolescentis* were fed to rats. The animal feeding trial was conducted at the Bureau of Nutritional Sciences at Health Canada (Ottawa, ON, Canada). Wistar rats (Health Canada, Animal Resources Division; n = 24) were fed an AIN93G purified diet (Research Diets, Inc., New Brunswick, NJ, USA) over a period of 7.5 weeks. Freeze-dried PPI-alginate microcapsules (0.5 g) were mixed with peanut butter (PB) prior to feeding. Animals were observed to consume all the PB-capsules per feeding time. Dosing of bacteria-containing

microcapsules was started 2.5 weeks after animal arrival. In order to ensure the nutritional adequacy of the supplement, an AIN mineral mix (Reeves *et al.*, 1993: 1.0 %; w/w) and AIN vitamin mix (1.5 %) were added to the PB. Capsules were first ground for 1 min using an ethanol-sterilized Black & Decker CBM210 coffee grinder (low speed setting) (Black & Decker Corp., Towson, Maryland, USA) to make smaller pieces to minimize the animal's ability to selectively graze the PB-capsule treatment. Animals were grouped according to diet, as follows: 1) AIN93G + PB; 2) AIN93G + PB + PPI-alginate microcapsules without encapsulated bacteria, and 3) AIN93G + PB + PPI-alginate microcapsules containing *B. adolescentis*. PB was added to the diet 6 days prior to the start of encapsulated bacterial treatment (day 1). The capsule intake was ceased on day 28. Reverse light/dark cycles were used: 7:00 pm – light, 7:00 am – dark. Animals were fasted for 4 h before feeding. Diet supplementation occurred at 11:00 am. Fecal pellets were collected from screens at 1 pm on days 0, 3, 8, 15, 23, 29, 36, 43, 51 and 57. Fecal pellets from each rat were preserved at -80°C until time of DNA extraction.

DNA was extracted from feces (0.1 g) taken from fecal pellets from two rats from each group using the Fast DNA SPIN Kit for Soil (MP Biomedicals LLC, Solon, OH, USA), quantified using a Qubit® 2.0 Fluorometer (Life Technologies™ Corp., Carlsbad, CA, USA) and amplified by the polymerase chain reaction (PCR), using primers *B_ado-F* and *B_ado-R* (see Table 3.1) (Junick and Blaut, 2012), specific for the *groEL* gene of *B. adolescentis*, and a TC-412 thermocycler (Techne Ltd., Duxford, Cambridge, UK). The reaction mix (50 µL) contained: *Taq* PCR Master Mix (Qiagen Sciences, Maryland, USA) – 20.0 µL, *B_ado-F* – 1.0 µL, *B_ado-R* – 1.0 µL, DNA template – 1.0 µL, nuclease-free ddH₂O (HyClone Laboratories Inc., Logan, Utah, USA) – 27.0 µL. The thermal cycle program was as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation (95°C, 45 s), annealing (62°C, 45 s) and elongation (72°C, 45 s), with a final elongation step of 10 min at 72°C. PCR products were analyzed using gel electrophoresis on a 1.5 % (w/v) agarose gel.

The DNA for qPCR was extracted from fecal samples using QIAamp DNA Stool Mini kit (Qiagen Sciences, Maryland, USA) according to manufacturer's protocol, with modifications. Quantitative PCR assays were conducted in polypropylene PCR plates in a ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All determinations were carried out in triplicate. The 10.0 µL qPCR reactions contained: 5.0 µL of 2X Brilliant III SYBR Green qPCR Master Mix (Agilent Technologies, Mississauga, ON), 1.0 µL of each primer (5.0 µM; Sigma-

Genosys Canada, Oakville, ON; Table 3.1), 2.0 µL molecular-grade water (Thermo Fisher Scientific, Waltham, MA, USA) and 1.0 µL of template DNA. PCR conditions were 3 min at 95°C, followed by 35-40 cycles of 95°C for 5 s and 15 s at the annealing temperature (Table 3.1). A melting curve analysis was conducted following the PCR reaction.

3.2.10 Statistics

Standard deviation, mean values and P-values within each group, as well as between groups, were determined using Two-Way ANOVA. The level of statistical significance was set at $p < 0.05$. All statistical analyses were performed using SPSS software (SPSS Inc., Ver. 17.0, 2008, Chicago, IL, USA). Graphs were created in SigmaPlot 11.0 (Systat Software, Inc., Chicago, IL).

Table 3.1. Primers and cycling conditions for PCR and qPCR.

| Primer name | Target | Primers sequence (5'-3') | Anneal. temp. | No. of cycles | Reference |
|----------------|---------------------------------|---------------------------|---------------|---------------|---------------------------------|
| <i>Bif582</i> | <i>Bifidobacterium spp.</i> | GGTGTGAAAGYCCATCGC | 58 | 35 | Health Canada (S. Brooks) |
| <i>Bif815</i> | | CACATCCAGCRTCCACCG | | | |
| <i>HDA1</i> | Total bacteria | ACTCCTACGGGAGGCAGCAGT | 60 | 35 | Walter <i>et al.</i> , 2000 |
| <i>HDA2</i> | | GTATTACCGCGGCTGCTGGCA | | | |
| <i>BiADO-1</i> | 16S rRNA <i>B. adolescentis</i> | CTCCAGTTGGATGCATGTC | 55 | 40 | Matsuki <i>et al.</i> , 2003 |
| <i>BiADO-2</i> | | CGAAGGCTTGCTCCCAGT | | | |
| <i>B_ado-F</i> | <i>groEL B. adolescentis</i> | CTCCGCCGCTGATCCGGAAGTCG | 72 | 40 | Junick and Blaut, 2012 |
| <i>B_ado-R</i> | | AACCAACTCGGCGATGTGGACGACA | | | |

3.3 Results and discussion

3.3.1 Survival of immobilized *B. adolescentis* cells in microcapsules exposed to synthetic stomach juice (SSJ)

Viability of *B. adolescentis* was monitored over a 2 h exposure to SSJ to determine the effect of encapsulation on their survival. Viability loss in planktonic cells was used as the control. Planktonic cells showed a significant decline in numbers over the first 30 min in SSJ, and a complete loss of viability following 90 min exposure ($D_{SSJ} = 11.46$ min; Fig. 3.1A). In contrast, all three types of encapsulated bacteria (PPI-alginate, PPI-*iota*-carrageenan, PPI-gellan gum) demonstrated significant ($p < 0.05$) retention of viability compared to planktonic cells (Fig. 3.1A). Total viability loss was similar for all types of microcapsules (~ 1.0-1.5 log reduction in CFU/g).

The effect of freeze-drying on the protective properties of microcapsules was also tested by repeating these determinations. All three types of freeze-dried microcapsules protected the cells from the detrimental effect of SSJ compared to planktonic cells (Fig. 3.1B). There was no significant difference in cell viability amongst different types of freeze-dried microcapsules ($p > 0.05$), similar to the trend observed for fresh capsules. D_{SSJ} -values for PPI-alginate, PPI-*iota*-carrageenan and PPI-gellan gum were 59.90, 56.80 and 46.20 min, respectively. Overall, the loss in viability following a 2 h exposure to simulated stomach juice was similar for all types of freeze-dried microcapsules (~ 2-2.5 log reduction in CFU/g).

3.3.2 Release of encapsulated cells

Following treatment for 2 h in SSJ, microcapsules were transferred to simulated intestinal fluid (SIF) for 3 h at 37°C. All formulations of both fresh and freeze-dried microcapsules released the majority of the encapsulated cells (Fig. 3.2A and B). Following treatment, PPI-*iota*-carrageenan microcapsules released the highest number of cell (7.4 log CFU/g) following incubation in SIF. Similar numbers of cells were released from the PPI-alginate microcapsules (7.0 log CFU/g). In contrast, PPI-gellan gum microcapsules released far fewer cells (4.0 log CFU/g), due to the greater resistance of PPI-gellan gum complex to degradation by SIF (Fig. 3.2A). Freeze-dried microcapsules released slightly more bacteria than fresh microcapsules (7.9, 7.5 and 5.3 log CFU/g for PPI-*iota*-carrageenan, PPI-alginate and PPI-gellan gum capsules, respectively; Fig. 3.2B).

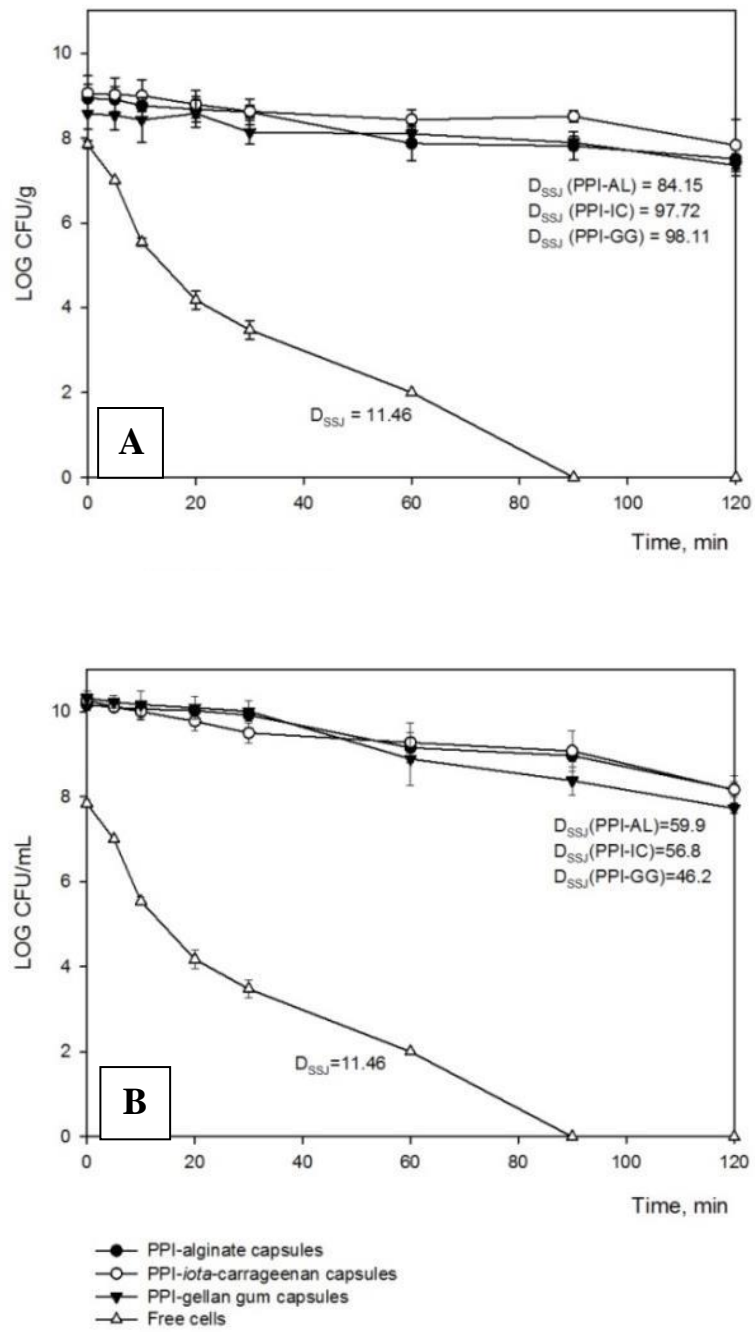


Figure 3.1. Decrease in *B. adolescentis* cell counts in fresh (A) and freeze-dried (B) microcapsules after 2 h in synthetic stomach juice.

3.3.3 Size of bacteria-containing capsules

In these experiments, all microcapsules were produced by extrusion of the polysaccharide-protein matrix mixed with bacterial cells into the cross-linking solution using an 18G needle. The resultant size of both fresh and freeze-dried PPI-alginate microcapsules was ~ 2 mm in diameter (Fig. 3.3A). Treatment with SSJ for 2 h at 37°C had no significant effect on capsule size (Fig. 3.3B). PPI-*iota*-carrageenan microcapsules were elliptical and decreased slightly in size from ~ 3 x 2 mm to ~ 2.5 x 1.5 mm after freeze-drying (Fig. 3A). When transferred to SSJ, these microcapsules became spherical and decreased in size to ~ 2 x 1.5 mm (fresh capsules) and ~ 2 x 2 mm (freeze-dried capsules; Fig. 3B). Fresh PPI-gellan gum microcapsules had an initial size of ~ 3.5 x 3 mm, which became reduced by ~ 3 x 2 mm after freeze-drying (Fig. 3.3A). After transferring to SIF, both fresh and freeze-dried PPI-alginate and PPI-*iota*-carrageenan microcapsules became dissolved in the solution after 3 h (Fig. 3.3C). PPI-gellan gum microcapsules of both types did not dissolve, but did change shape and size, becoming spherical and ~ 3 mm in diameter. The dissolution of PPI-alginate and PPI-*iota*-carrageenan microcapsules under simulated conditions of lower GIT might also be an asset due to the increased amount of viable bacteria released.

3.3.4 CLSM imaging of the internal structure of bacteria-containing capsules

All three types of microcapsules (both fresh and freeze-dried) were examined using confocal microscopy to characterize the internal capsule structure and spatial distribution of cells. CLSM images of stained (SYTO-9), bisected fresh microcapsules revealed that the cells were evenly distributed within the microcapsules (Fig. 3.4A). Cells were immobilized on the surface of pea protein particles (which emitted green autofluorescence) and within the unstained polysaccharide (transparent background; Fig. 3.4A). In freeze-dried microcapsules (Fig. 3.4B), protein globules were slightly deformed and surrounded by layers of polysaccharide containing entrapped bacteria, forming pores separated by protein-polysaccharide walls.

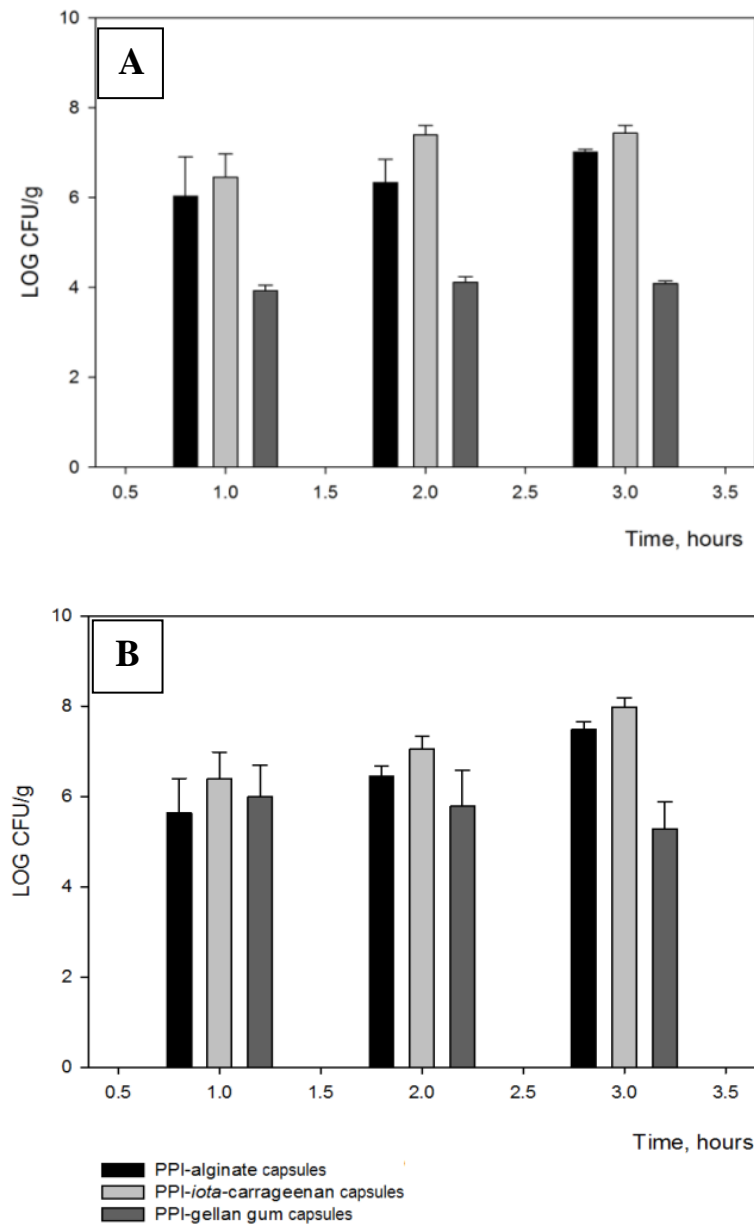


Figure 3.2. Release of *B. adolescentis* cells from fresh (A) and freeze-dried (B) microcapsules after 2 h in simulated intestinal fluid.

3.3.5 Animal feeding trial

Genus-specific and species-specific oligonucleotide primer sets targeting the *Bifidobacterial* 16S rRNA gene and the *GroEL* gene of *B. adolescentis* were used to confirm the presence of DNA from this organism in the feces of rats fed PPI-alginate-immobilized cells. Universal primers were used to determine the total amount of bacteria in samples (Table 3.1).

Fecal community DNA was extracted from rat feces on days 0 (the first day of PPI-alginate microcapsules intake), 8, 29 (the first day after elimination of probiotic supplement), 36, 43 and 57. The results of PCR with *B. adolescentis*-specific primers, followed by gel electrophoresis, demonstrated an increased amount of *B. adolescentis* in the GIT of rats that were fed PPI-alginate microcapsules containing bacteria on days 8 and 29 (data not shown). However, there were no amplicons that corresponded to *B. adolescentis* present in gels from days 36, 43 and 57, which suggests that the elimination of delivered bacteria from animal GIT occurred within one-week post-supplementation.

Quantitative PCR data showed an increase in the number of gene copies per ng (both 16S rRNA and *groEL*) of *B. adolescentis* in DNA extracted from fecal samples of rats receiving the encapsulated bacteria supplement (Fig. 3.5). The samples used in this experiment were collected on day 29. On average, *Bifidobacteria* made up 1-1.5 % of the total community 16S rRNA gene content (3.5-4.0, compared to 6.1 log 16S rRNA gene copies per ng of DNA) (Fig. 3.5) and there was no difference in 16S rRNA gene copies between dietary treatments (Fig. 3.5). *Bifidobacterium adolescentis* encompassed ~ 16-28 % of total fecal *Bifidobacteria* in the rat group that received the encapsulated bacteria supplement, depending on the primer set used for quantification (Fig. 3.5). There were no *B. adolescentis* detected by qPCR in samples taken from animals in groups that did not receive the encapsulated bacteria. Seven days after encapsulated *B. adolescentis* supplementation ended, DNA from this species was no longer detectable in feces. The amount of *B. adolescentis groEL* gene copies was lower than that of 16S rRNA due to differences in gene copy numbers per cell: only one copy is present for the chaperonin gene whereas 5 copies exist for the rRNA gene.

Previously, Wood *et al.* (2010) found that microcapsules composed solely of alginate (1.0 % w/v) were unable to provide any significant protection to *B. adolescentis* ATCC 15703 during a 2 h incubation in simulated gastric juice. The inability to protect the cells was attributed to the presence of large pores (~ 400 µm) on the surface of these capsules. This is in marked contrast to Lee and Heo (2000) who reported only a 2 log CFU/mL decrease in viable cell numbers of *Bifidobacterium*

longum encapsulated in 2.0 % (w/v) alginate and held under similar conditions. This difference might be attributed to a higher degree of acid tolerance of *B. longum*, as different species of bifidobacteria have varying sensitivities to acidic conditions (Waddington *et al.*, 2011). In addition, this may also reflect difference in the size of bead. Generally, the larger the bead, the greater the protective effect of immobilization.

Our findings from *in vitro* survival tests are supported by other investigations. Klemmer *et al.* (2011) reported an ~ 1.0 log CFU/mL reduction of viable cell counts for PPI-alginate microcapsules (2.0 % w/v and 0.5 % w/v, respectively) exposed to different simulated stomach conditions – synthetic gastric juice (0.08 M HCl with 0.2 % (w/v) NaCl; pH 2). Khan *et al.* (2013) reported a ~ 2.0-2.6 log CFU/g reduction of viable *B. adolescentis* cells entrapped in 2.0 % (w/v) PPI – 0.5 % (w/v) alginate microcapsules after 2 h in synthetic stomach juice (pH 1.8). Interestingly, increasing of the concentration of PPI to 4.0 % lead to lower viable counts of *B. adolescentis* after 2 h in synthetic gastric juice – 3.6 log CFU/mL reduction (Wood, 2010). The comparative analysis of release data supports the conclusion that PPI-alginate and PPI-*iota*-carrageenan microcapsules provide for the more effective delivery of live bacteria than PPI-gellan gum capsules, since these two types of microcapsules were able to release up to 70-79 % of entrapped bacteria under simulated lower GIT conditions. In this case, the higher number of released cells may be explained by the increased susceptibility of freeze-dried probiotic microcapsules to degradation for 3 h in SIF.

A possible reason for the dissolution of PPI-*iota*-carrageenan microcapsules might be disruption of electrostatic bonds existing between protein and polysaccharide. Since PPI has an isoelectric point (pI) of ~ 4.5, at pH values < pI, the protein would assume a net-positive charge; whereas, at pH values > pI, the protein would carry a net-negative charge. In contrast, all polysaccharides used in this study would carry a negative charge at pH values > 1.88 (pK_a of –COO[–]) in the case of gellan gum and alginate, and > 2.00 (pK_a of –SO^{3–}) in the case of *iota*-carrageenan. Where the polysaccharide pK_a < pH < pI (protein), both components would be oppositely-charged, and thus they would be electrostatically-attracted to each other (Ganzevles, 2007). Hence, in SSJ at pH 1.8, complexes would remain intact due to both Ca²⁺ stabilization and attractive protein-polysaccharide interactions. However, under simulated intestinal conditions (pH 6.5), both protein and polysaccharide would have the same charge and thus would repel each other.

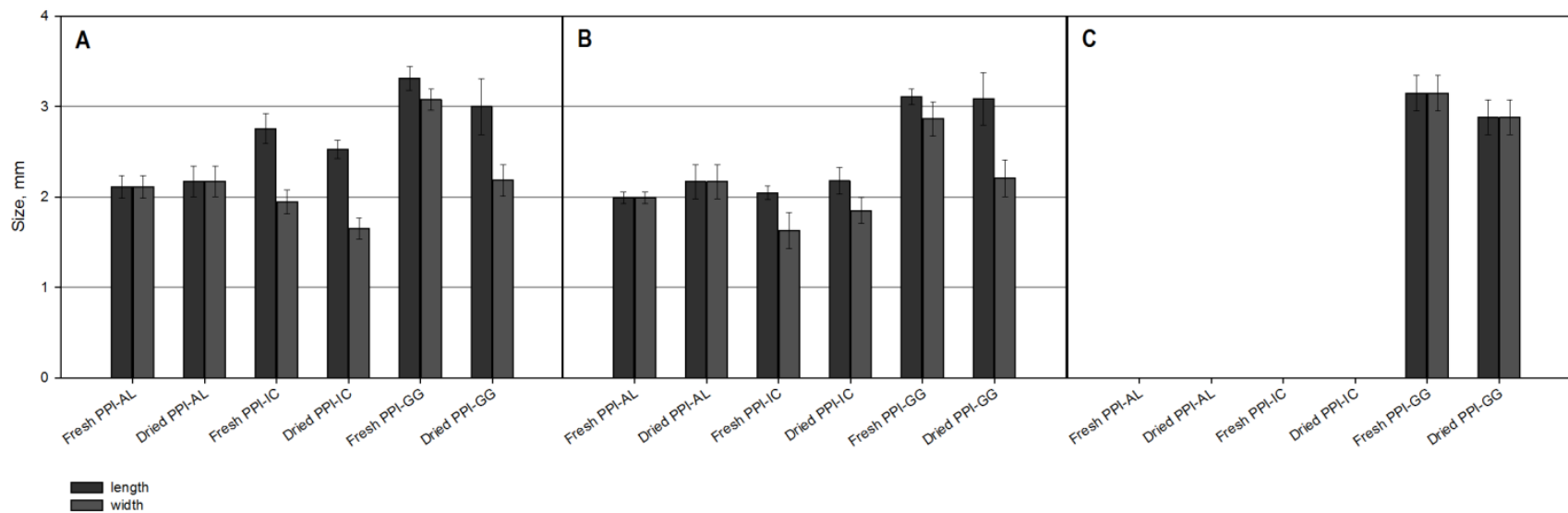


Figure 3.3. Size of probiotic capsules. A. Initial size. B. Size after 2 h in synthetic stomach juice. C. Size after 2 h in synthetic stomach juice, followed by 3 h in simulated intestinal fluid. Abbreviations: PPI-AL – pea protein isolate-alginate, PPI-IC - pea protein isolate – *iota*-carrageebnan, PPI-GG - pea protein isolate –gellan gum.

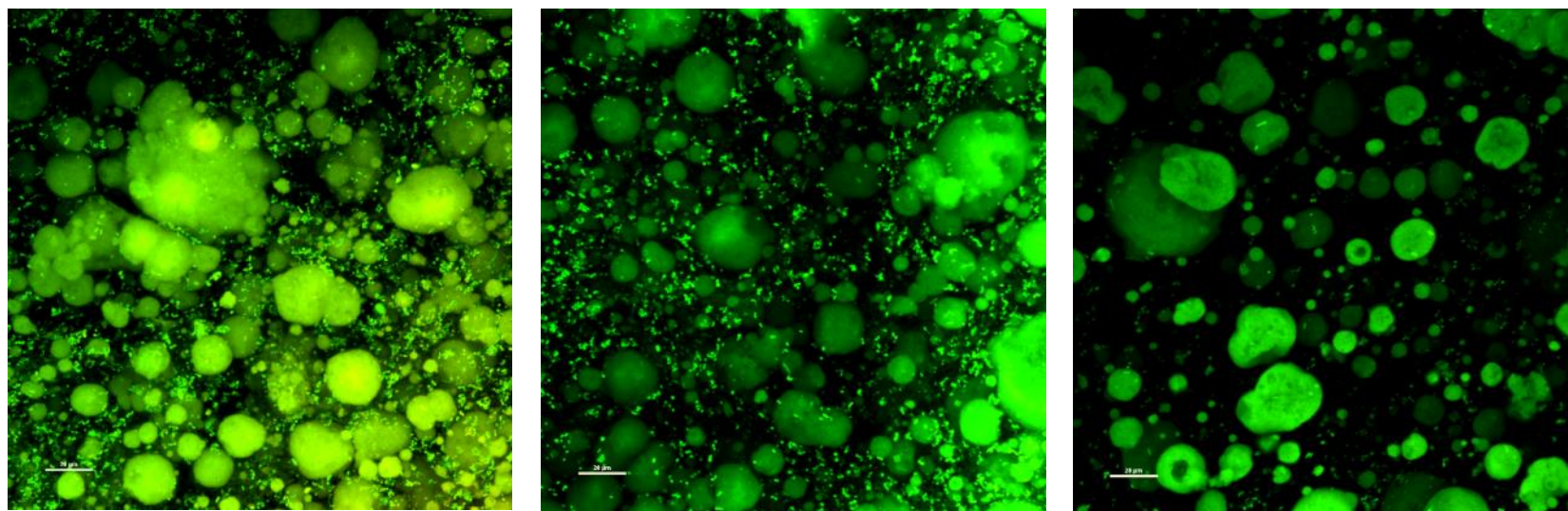


Figure 3.4A. Maximum intensity projections of a series of CLSM images of fresh probiotic capsules, stained with SYTO-9. From left to right: PPI-alginate, PPI-*iota*-carrageenan and PPI-gellan gum capsule structure. Images were collected over a 50 μm depth interval, z -step – 0.5 μm (1 μm for PPI-alginate capsules), scale bar – 20 μm. Bacteria are seen as the smallest detectable rod-shaped or bifurcated particles fluorescing in the green wavelength. Globular macro-structures on the images are particles of PPI, emitting autofluorescence.

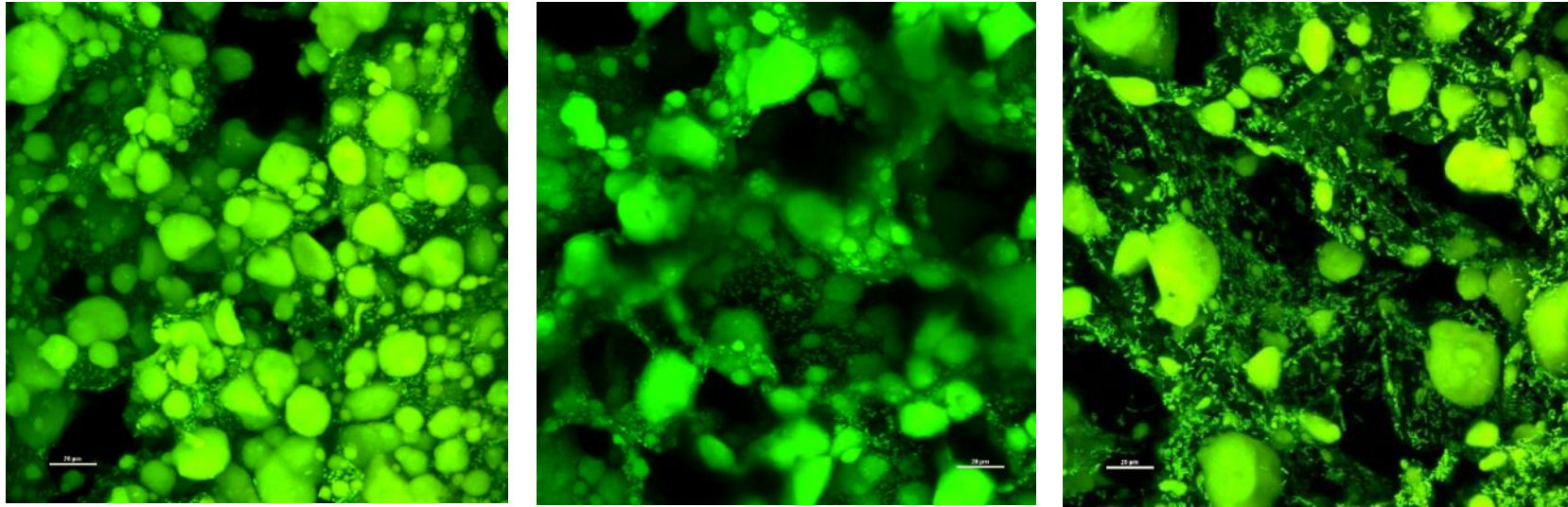


Figure 3.4B. Maximum intensity projections of a series of CLSM images of freeze-dried probiotic capsules, stained with SYTO-9. From left to right: PPI-alginate, PPI-*iota*-carrageenan and PPI-gellan gum capsule structure. Images were collected over a 50 μm depth interval, z -step – 0.5 μm, scale bar – 20 μm. Bacteria are seen as the smallest detectable rod-shaped or bifurcated particles fluorescing in the green wavelength. Globular macro-structures on the images are particles of PPI, emitting autofluorescence.

It was hypothesized in this case that the structural integrity of a capsule would be maintained by the calcium-stabilizing polysaccharide network. In comparison with other polysaccharides tested, *iota*-carrageenan had a greater net-negative charge (due to higher electronegativity of its sulphate groups), which would lead to higher repulsive forces within the microcapsule and, consequently, disruption of the calcium-carrageenan network followed by release of up to 79 % of entrapped bacteria into the solution (Fig. 3.2). These findings are supported by the studies of polymer and drug release characteristics from alginate capsules (Kim and Lee, 1992; Sugawara *et al.*, 1994; Chen *et al.*, 2004, 2007). At low pH (e.g., gastric conditions), the surface of alginate capsules would become porous and insoluble (George and Abraham, 2006). In contrast, in higher pH solutions (e.g., intestinal conditions) this so-called “alginic acid skin” becomes soluble, and alginate starts to swell, which, in the case of probiotic capsules might aid in the controlled release of bacterial cells under gastric and intestinal conditions (Kimura, 1993).

In general, low-density dried polysaccharide gels, having an open cellular structure with thin walls, such as alginate, are considered very fragile (Nussinovitch *et al.*, 2000). The stability of such gels can be preserved by using freeze-drying as a preferable method for dehydration, as it results in dry gels with improved mechanical properties (Nussinovitch *et al.*, 1993). The addition of pea protein to polysaccharide gels also considerably improved their stability under acidic conditions, allowing effective protection of immobilized bacteria.

A honeycomb-like ultrastructure has been previously described by Khan *et al.* (2013) and Wood (2010), based on scanning electron microscopy (SEM) and cryo-SEM of PPI-alginate capsules. Both these techniques require the fixation of samples followed by dehydration, which might explain similarities in images of fresh capsules acquired by SEM, cryo-SEM and CLSM of freeze-dried capsules (Liu *et al.*, 2008; Serp *et al.*, 2002). Cryo-SEM images of capsules made of sodium alginate alone also revealed a honeycomb-like internal structure (Wood, 2010). However, as evident from our survival studies, these pores apparently do not facilitate the diffusion of simulated stomach juice into the capsules, as the viability of bacteria within both freeze-dried- and fresh microcapsules was approximately equal.

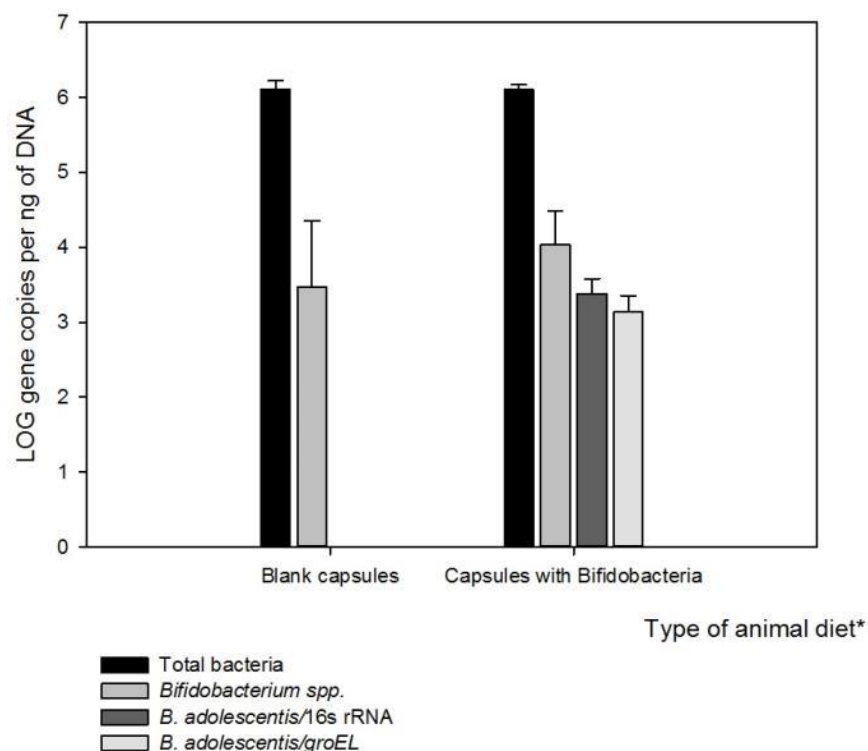


Figure 3.5. The number of gene copies per ng of DNA, extracted from fecal samples of rats, receiving different types of diet (day 29).

*The DNA for qPCR analysis was extracted from fecal samples of two groups of rats: receiving blank PPI-alginate microcapsules or PPI-alginate microcapsules with bifidobacteria.

Capsule size is a key parameter that likely would dictate the range of food products in which a particular size of capsule could be used. In general, the smaller the capsule, the better the organoleptic properties of the product. However, decreasing the capsule size leads to poorer protection of entrapped bacteria (Lee and Heo, 2000; Chandramouli *et al.*, 2004). The extrusion method used in the present study does not require any additional raw materials, such as emulsifiers, and allows the production of capsules of relatively consistent size, depending on the needle gauge and concentration of polymers used (Wood, 2010; Klemmer *et al.*, 2011; Gbassi and Vandamme, 2012; Khan *et al.*, 2013). A further advantage of the extrusion method is the gentle conditions of capsule formation, which facilitates maintenance of high cell viability (80–95 %)

(Krasaekoopt *et al.*, 2003). Evidence from the literature suggests the size range of capsules that may be produced by extrusion to be approximately 1-3 mm in diameter. Klemmer *et al.* (2011), working with 2.0 % (w/v) PPI – 0.5 % (w/v) alginate solution, produced microcapsules with a diameter of 1.65-1.89 mm and 2.01-2.25 mm, using 27G and 20G needles, respectively. Similar results were achieved by Khan *et al.* (2013), who used 16, 18, 19, 23 and 27G needles for the extrusion of the same type of microcapsules and reported mean capsule sizes ranging from 1.23 mm (27G) to 2.79 mm (16G). In general, while smaller sized microcapsules would be preferable for potential use in food products, it would result in reduced protection for immobilized bacteria.

It has been widely-reported that in order to have a health-promoting effect, the concentration of probiotic bacteria in a product should be at least 10^6 CFU per ml or g (Kailasapathy and Chin, 2000). Culture-dependent methods for bacterial enumeration are prone to false-positive and false-negative results and cannot be applied to the quantification of many bacterial species due to the absence of a suitable medium, which makes culture-independent techniques, such as PCR, a useful tool used for this purpose (Giraffa and Carminati, 2008). Both species-specific and multiplex PCR have already been successfully used for the quantification of bacteria (including *B. adolescentis*) in human and animal feces (Wang *et al.*, 1996; O’Sullivan, 1999). In a study by Satokari *et al.* (2001), a group of human subjects were administered *Bifidobacterium lactis* at a concentration of about 3×10^{10} CFU per day for two weeks. The presence of *B. lactis* during the period of administration was detected in feces by PCR/gel electrophoresis techniques. However, no *B. lactis* were detected after the administration had been ceased (Satokari *et al.*, 2001).

In general, the majority of research on probiotic delivery tools has focused on the indirect assessment of the efficacy – through disease treatment and presence of beneficial effects on health. Whereas, there remains a considerable lack of scientific research that has focused on the assessment of probiotic delivery into the GIT of model animals, receiving daily intake of bacteria of interest, entrapped within microcapsules or capsules. Though indirect approaches allows the evaluation of the beneficial effect of the supplement on health, it is essential to study the functional mechanisms of probiotic delivery tools. There is a need for research of the controlled release of bacteria from capsules, which requires testing of new materials and techniques. Protecting and releasing bacteria at specific sites could be useful in targeting a specific health problem: e.g., the immune response could be altered and improved by delivery of beneficial bacteria into the small intestine, while the

antipathogenic properties of probiotics would have the greatest effect in the colon (Cook *et al.*, 2012).

The type of microorganisms used for the testing of the efficacy of a certain protection technique (e.g. capsulation or using of bacteria-containing beads) also plays an important role. Widely-used probiotic strains of lactobacilli are less pH-sensitive than bifidobacteria, which reportedly lack acid tolerance response mechanisms and, therefore, require enhanced protection during the passage through the stomach (Waddington *et al.*, 2010; Sahadeva *et al.*, 2011). The use of acid-sensitive strains would provide additional proof of the efficacy of the chosen delivery tool and would make it easier to deliver significant amounts of potentially-beneficial, but acid-sensitive, bacteria into lower GI tract.

Encapsulation has been proven by many to be a potentially useful tool for the delivery of probiotic bacteria into the lower GIT in sufficiently high numbers. However, molecular techniques should be used to confirm the delivery of encapsulated bacteria into specific sites of the GIT as well as to assess any alterations this might bring to the microbiota of the host. Use of a variety of materials with gelling properties and ability to interact with polysaccharides to form protective microcapsules in conjunction with acid-sensitive bacteria as a mean of testing the capsule efficacy would make it possible to target an effective encapsulation technique for specific health applications.

3.4 Conclusions

A series of *in vitro* experiments employing simulated conditions of the human GIT revealed that PPI-polysaccharide microcapsules containing acid-sensitive *B. adolescentis* have potential for protecting these bacterial cells from the detrimental effects of the upper GIT, with subsequent release of significant amounts of bacteria in the lower GIT. The overall cell count reduction after 2 h in SSJ at 37°C was similar for all types of capsules, and was equivalent to a loss of ~ 1.0-1.5 log viable CFU/g for fresh microcapsules and ~ 2.0-2.5 log CFU/g for freeze-dried capsules. However, the subsequent release of bacteria from the microcapsules was highest for PPI-*iota*-carrageenan and PPI-alginate capsules; whereas, 70 – 74 % of the total amount of immobilized cells was released from fresh microcapsules and 75 – 79 % of the immobilized cells were released from freeze-dried capsules. Capsules formulated with PPI-gellan gum released the fewest cells, with only ~ 4.0 log CFU/g detected following incubation in SIF.

The molecular analysis of fecal samples recovered from an *in vivo* rat feeding study confirm that PPI-alginate microcapsules are an effective tool to deliver bacteria such as probiotics into the lower gut. During the period of feeding encapsulated *B. adolescentis*, DNA extracted from rat feces showed an increased amount of *B. adolescentis*, a finding together with the results of *in vitro* survival tests, reveals that PPI-alginate microcapsules indeed protect bacterial cells from highly-acidic conditions of the stomach with subsequent release of bacteria from the microcapsules in the intestine.

3.5 Acknowledgements

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3.6 Preface to next chapter

From the results of the first study, pea-protein alginate was chosen as the best microcapsule type of all tested suitable for the delivery of oxygen- and acid-sensitive bacteria into an animal GIT. The next step would be evaluating the effects that these microcapsule wall materials would have on their ability to deliver and protect probiotics into the GI tract in an actual animal system. Accordingly, *Citrobacter rodentium* – induced colitis in mice was chosen as a model system to study the efficacy of probiotics encapsulated in PPI-alginate. In preparation for animal studies, our laboratory collection of lactobacilli strains was screened for antagonistic properties against the pathogen *C. rodentium* in order to choose a candidate for encapsulation.

4 TESTING OF ANTAGONISTIC PROPERTIES OF PROBIOTIC STRAINS AGAINST *CITROBACTER RODENTIUM*

4.1 Abstract

Bacterial strains with potential probiotic properties were tested for their ability to inhibit the growth of *Citrobacter rodentium*, a pathogen causing colitis in mice. One or several probiotic strains, selected in this study for their ability to inhibit the growth of *C. rodentium in vitro*, would have been encapsulated in a PPI-alginate matrix and tested *in vivo* in mice for their ability to prevent or alleviate the symptoms of colitis like symptoms (e.g. colonic epithelial cell hyperplasia) caused by this pathogen. In *in vitro* antagonism tests as well as in co-aggregation experiments none of the strains tested proved useful for inhibiting the growth of this pathogen. However, two strains – *Lactobacillus rhamnosus* R0052 and *Lactococcus lactis* ATCC 11454 – showed a strong ability to attach to HEp-2 cells *in vitro*. Based on these findings as well as on previous work in the field, a mixture of commercial lactobacilli strains, including *L. rhamnosus* R0052, was chosen for the *in vivo* testing of capsule efficiency.

4.2 Introduction

A pathogenic microorganism *C. rodentium* is used to model several human intestinal disorders, such as Crohn's disease, ulcerative colitis and colon tumorigenesis (Higgins *et al.*, 1999; Chandrakesan *et al.*, 2013). Its mechanisms of pathogenesis resemble those of enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC), which are leading causes of diarrhea, haemorrhagic colitis and haemolytic uremic syndrome, thus it is often used to study the molecular basis of infections, caused by these pathogens (Crepin *et al.*, 2016; Flowers *et al.*, 2016; Lackraj *et al.*, 2016).

Several studies showed that a mixture of two probiotic strains – *L. rhamnosus* R0011 and *L. helveticus* R0052 (used in commercial Lacidofil[®] preparation; Lallemand Health Solutions, Montreal, QC, Canada) – is able to inhibit *C. rodentium* infection *in vivo* by downregulation of the

expression of pro-inflammatory cytokines and reduction of the attachment of the pathogen to colonocytes (Johnson-Henry *et al.*, 2005; Gareau *et al.*, 2010; Rodrigues *et al.*, 2012). *In vitro* experiments testing these strains against multiple pathogens were conducted using cell cultures, such as T84 and Caco-2, and proved that probiotics were effectively inhibiting pathogen attachment to cells (Sherman *et al.*, 2005; Atassi *et al.*, 2006; Wine *et al.*, 2009). Additionally it has been previously shown that the growth of *C. rodentium* can be inhibited *in vitro* by co-culturing with bacteria, producing lactic acid, as well as by the addition of ascorbic acid to the culture medium (Idoui, 2014; Abu-Ghazaleh, 2006; Pasteris *et al.*, 2011).

The potential antagonistic activity of several LAB strains against the pathogen was tested in a series of *in vitro* tests. Additionally, the ability of probiotic bacteria to coaggregate with *C. rodentium* was tested as an important trait that could potentially inhibit the pathogen activity, including its attachment to epithelial cells.

Citrobacter rodentium is an attaching and effacing (A/E) type of pathogen which is able to attach to the apical cell membrane of the cecal and colonic epithelia, forming a non-invasive structure (“pedestal”) on the host cell (Bhinder *et al.*, 2013). In order to test possible inhibition of such attachment by a set of lactobacilli strains, HEp-2 cells were used in *in situ* experiments, since it has been shown previously that *C. rodentium* forms “pedestals” on the surface of these cells (Frankel *et al.*, 1994; Newman *et al.*, 1999). The cell line have been established via HeLa cell contamination and is positive for the presence of human papilloma viral DNA. The specific strain used was HEp-2 ATCC CCL-23.

The hypothesis was as follows: probiotic strains having the ability to inhibit the growth of *C. rodentium* (Schauer *et al.*) ATCC 51459 *in vitro* will be identified based on a series of bacterial growth antagonism assays, co-aggregation tests and their ability to inhibit the attachment of *C. rodentium* to human cells *in vitro*.

4.3 Methods

4.3.1 Bacterial strains

The following strains from our laboratory collection were tested: *Lactobacillus delbrueckii* ATCC 9649, *Lactobacillus fermentum* NRRL B4524, *Lactobacillus fermentum* Beierinck ATCC 14931, *Lactobacillus fermentum* Beijerinck ATCC 11739, *Lactobacillus helveticus* ATCC 15009, *Lactobacillus maltaromicus* (Miller *et al.*) ATCC 27865, *Lactobacillus plantarum* ATCC 14917,

Lactococcus lactis ATCC 11454, *Lactobacillus rhamnosus* ATCC 1469, *Lactobacillus rhamnosus* ATCC 15820, *Pediococcus acidilactici* ATCC 25740, *Pediococcus parvulus* 2.6 and *Pediococcus pentosaceus* ATCC 33316. Additionally *L. rhamnosus* R0011 and *L. helveticus* R0052 were received from Lallemand Health Solutions (Montreal, QC, Canada) and tested as well.

4.3.2 Antagonism tests

Antagonistic activity of bacteria against *C. rodentium* was determined using a cross-streak method (Kotikalapudi (2009) with modifications). In brief, strains of lactobacilli were streaked on Plate Count (PC) agar plates, after which *C. rodentium* was streaked perpendicular to LABs. Plates were then incubated at 37°C for 48 h and the distance between streaks measured (n = 6).

For direct antagonism plate test (Touré *et al.*, 2003), 2 µL of overnight culture of LAB isolates, grown in MRS broth, were spotted on 1.5 % MRS agar containing 2 g/L of sodium bicarbonate to avoid interference from acid production. Plates were dried for 30 min at room temperature and incubated anaerobically at 37°C for 18 h, then overlaid with 10 ml of PC containing 0.8 % agar at 45°C that was seeded with 1 % (v/v) of an active overnight culture of *C. rodentium*. Overlaid plates were incubated aerobically at 37°C for 18 h. Testing was carried out in triplicate. Results (zones of inhibition) were reported as the distance (mm) from the edge of the LAB colony to the outer edge of the inhibition zone.

The ability of cell-free culture supernatants to inhibit the growth of pathogens was tested utilizing well diffusion assay (Meshref and Korný, 2014). LAB-inoculated 24 h broths were centrifuged at $12000 \times g$ for 10 min (Microfuge 16; Beckman Coulter, Brea, CA, USA); the pellet was discarded and the supernatant was neutralized to pH 6.2 with 1M NaOH and filter sterilized through 0.45 µm pore size filter. Aliquots of 150 µl of supernatants were added into well, cut in the MRS agar. Well diameter was 9 mm; the bottom of each well was sealed beforehand with a drop of liquid MRS agar. Inhibition zone diameter was measured after 24 h incubation at 37°C (n = 3).

Acid production by individual LAB strains was also observed on MRS agar with bromocresol purple and assessed in 24 and 48 h after inoculation as being low (+), medium (++) or high (+++).

4.3.3 Coaggregation assay

The coaggregation assay was performed as described in Kotikalapudi, 2009. Overnight cultures were harvested by centrifugation at $3,250 \times g$ (Microfuge 16; Beckman Coulter) for 15 min

at 4°C and washed once with PBS and resuspended in the same buffer to an $OD_{600} = 0.250$ (BioSpectrophotometer; Eppendorf, Hamburg, Germany). Aliquots of 0.5 mL of *C. rodentium* suspension was added to each of the LAB suspensions in sterile test tubes and mixed for 10s on a vortex mixer, and then incubated at 37°C for 24 h without agitation, aerobically or anaerobically. The absorbance (A_{600}) of the cell suspension was measured after 2, 4 and 24 h of incubation. The co-aggregation (%) was calculated according to the following equation: $[(A_{pat} + A_{probio}) - (A_{mix}) / (A_{pat} + A_{probio})] \times 100$, where, A_{pat} and A_{probio} represent OD_{600} of the pathogen (pat) and probiotics (probio) suspensions at time 0 min and A_{mix} represents OD_{600} of the mixed bacterial suspension at different times. Additionally co-aggregation abilities of bacteria were screened by visual observation, and the degree of aggregation was recorded on a scale 0 to 4+: a score of 0 for no visible aggregates in the cell suspension, 1+ for small uniform aggregates in the suspension, 2+ for aggregates that were easily seen but did not settle, 3+ for large aggregates which settled and left some turbidity in the supernatant fluid, and 4+ for large aggregates which settled and left clear supernatant fluid. Auto-aggregation of LAB strains was also tested by using monocultures and was used as a control to measure co-aggregation, which was considered to occur when the score in the bacterial mixtures was greater than the auto-aggregation score of either strain in monoculture.

4.3.4 Attachment of *C. rodentium* and *lactobacilli* to human cells

The human epithelial laryngocarcinoma (HEp-2) cells were propagated in Dulbeccos's Modified Minimum Essential medium (DMEM; Thermo Fisher Scientific, Madison, WI, USA) with 10 % (v/v) fetal bovine serum (Thermo Fisher Scientific, Madison, WI, USA) in 75 mL flasks at 37°C in a cell incubator maintained under 5 % CO_2 atmosphere (Fisher Scientific Isoterm CO_2 incubator, Thermo Fisher Scientific, Madison, WI, USA). Cells were reseeded every time confluence reached ~ 95 %. For this, cells were trypsinized with TrypLE Express (Thermo Fisher Scientific, Madison, WI, USA), washed from flasks using sterile DMEM, transferred to 50 mL tubes, centrifuged at 1300 rpm for 5 min, and then transferred to new flasks with fresh DMEM. Adhesion assays were performed according to Bai *et al.* (2012) with minor modifications, using *E. coli* HB101 as negative control. In brief, HEp-2 cells were seeded into 24-well plates 24 h before infection at a density of 5×10^4 cells per well. Before infection the medium was replaced with new DMEM medium containing 1% mannose. A mixture with a MOI (number of bacteria per number of mammalian cells) of 100:1 was added to wells and allowed to adhere for 3 h at 37°C in 5 % CO_2 .

After incubation, each well was rinsed five times with PBS, cells were fixed in pre-cooled to -20°C methanol for 15 min and stained with a mixture of 0.5 % safranin (Safranin T; Sigma-Aldrich, Oakville, ON, Canada), 0.5 % methylene blue (Sigma-Aldrich) and 0.4 % trypan blue (Mediatech Inc., Manassas, VA, USA). After staining wells were washed once with 1 × PBS and examined using inverted microscope (Olympus IX71; Olympus corp., Tokyo, Japan) under brightfield illumination at a magnification of × 400. An adhesion index (< 1, > 1, < 50 and > 50) was determined by examining 10 visual fields per well. Experiments were repeated three times in duplicate.

4.4 Results

Most of the tested LAB strains showed no antagonism towards *C. rodentium*, as was evident from growth using the cross-streak method. In contrast, direct antagonism testing and the well-diffusion assay did show some inhibition of the growth of this pathogen. However, those data were not considered sufficiently significant to warrant using these strains in *in vivo* studies (Table 4.1).

Though multiple species of LAB are able to coaggregate with pathogens (Ekmekci *et al.*, 2009; Kotikalapudi, 2009; Twetman *et al.*, 2009; Keller *et al.*, 2011; Younes *et al.*, 2012), there is a very limited data available on the coaggregation of *C. rodentium* with other bacteria (Stevens *et al.*, 2015). Co-aggregation experiments did not provide positive results for any of the strains tested (data not shown). None of the LAB strains were able to co-aggregate with *C. rodentium*, which was evident from both optical density measurements, which did not change significantly in time, and from visual analysis of the culture mixtures – no flocculation was observed.

Others have previously reported that *C. rodentium* was cytotoxic towards HEp-2 cells, though the ability to attach to cell surface differed significantly among isolates (Bai *et al.*, 2012). In this study, there was no attachment observed of *C. rodentium* to human cells (Figure 4.1), which made it impossible to test whether LAB inhibit the attachment of the pathogen. However, two of the lactobacilli strains – *L. helveticus* R0052 and *L. lactis* ATCC 11454 showed a strong ability to attach to HEp-2 cells with adhesion indices > 50 and < 50, respectively (Fig. 4.1). Sherman *et al.* (2005) have previously reported that *L. helveticus* R0052 has the ability to adhere to host epithelial cells, and, when using in a mixture with *L. rhamnosus* R0011, is able to reduce the binding of both *E. coli* O157:H7 and *E. coli* E2348/69 to HEp-2 and T84 cells. Since *C. rodentium* utilizes mechanisms of epithelial cell attachment that are very similar *E. coli*, it can be hypothesized that the adhesion of

lactobacilli to receptors on the surface of human cells could decrease numbers of available binding sites for this enteric pathogen.

The ability of *L. lactis* ATCC 11454 to attach to HEp-2 cells could also be considered a beneficial trait, which, to the best of my knowledge, has not been reported before. According to previous research this strain was able to inhibit the growth of *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium in vitro* (Millette *et al.*, 2004).

Table 4.1. Results of antagonism tests

| Strain | Cross-streak method, mm | Direct antagonism test, mm | Well-diffusion assay, mm | Acid production at 24 h |
|---|-------------------------|----------------------------|--------------------------|-------------------------|
| <i>Lactobacillus delbrueckii</i> ATCC 9649 | 0 | 1.25 ± 0.25 | 17.17 | + |
| <i>Lactobacillus fermentum</i> B4524 | 0 | 1.50 ± 0.00 | 17.50 | ++ |
| <i>Lactobacillus fermentum</i> Beierinck ATCC 14931 | 0 | 2.00 ± 0.00 | 16.33 | ++ |
| <i>Lactobacillus fermentum</i> Beijerinck ATCC 11739 | 0 | 2.00 ± 0.50 | 16.83 | ++ |
| <i>Lactococcus lactis</i> ATCC 11454 | 0 | 1.50 ± 0.00 | 17.50 | ++ |
| <i>Lactobacillus helveticus</i> ATCC 15009 | 0 | 1.17 ± 0.29 | 16.33 | ++ |
| <i>Lactobacillus maltaromicus</i> Miller <i>et al.</i> ATCC 27865 | 0 | 1.83 ± 0.29 | 17.00 | ++ |
| <i>Lactobacillus plantarum</i> ATCC 14917 | 0 | 4.00 ± 0.50 | 17.83 | +++ |
| <i>Lactobacillus rhamnosus</i> ATCC 1469 | 0 | 2.00 ± 1.00 | 16.83 | ++ |
| <i>Lactobacillus rhamnosus</i> ATCC 15820 | 0 | 2.50 ± 0.50 | 17.83 | + |
| <i>Lactobacillus rhamnosus</i> R0011 | 0 | 2.00 ± 0.05 | 17.00 | + |
| <i>Lactobacillus helveticus</i> R0052 | 0 | 1.15 ± 0.05 | 16.00 | + |
| <i>Pediococcus acidilactici</i> ATCC 25740 | 3.33±2.25 | 1.67 ± 0.58 | 16.00 | ++ |
| <i>Pediococcus parvulus</i> 2.6 | 0 | 1.83 ± 0.29 | 15.33 | ++ |
| <i>Pediococcus pentosaceus</i> ATCC 33316 | 3.16±1.57 | 2.17 ± 0.29 | 15.83 | ++ |

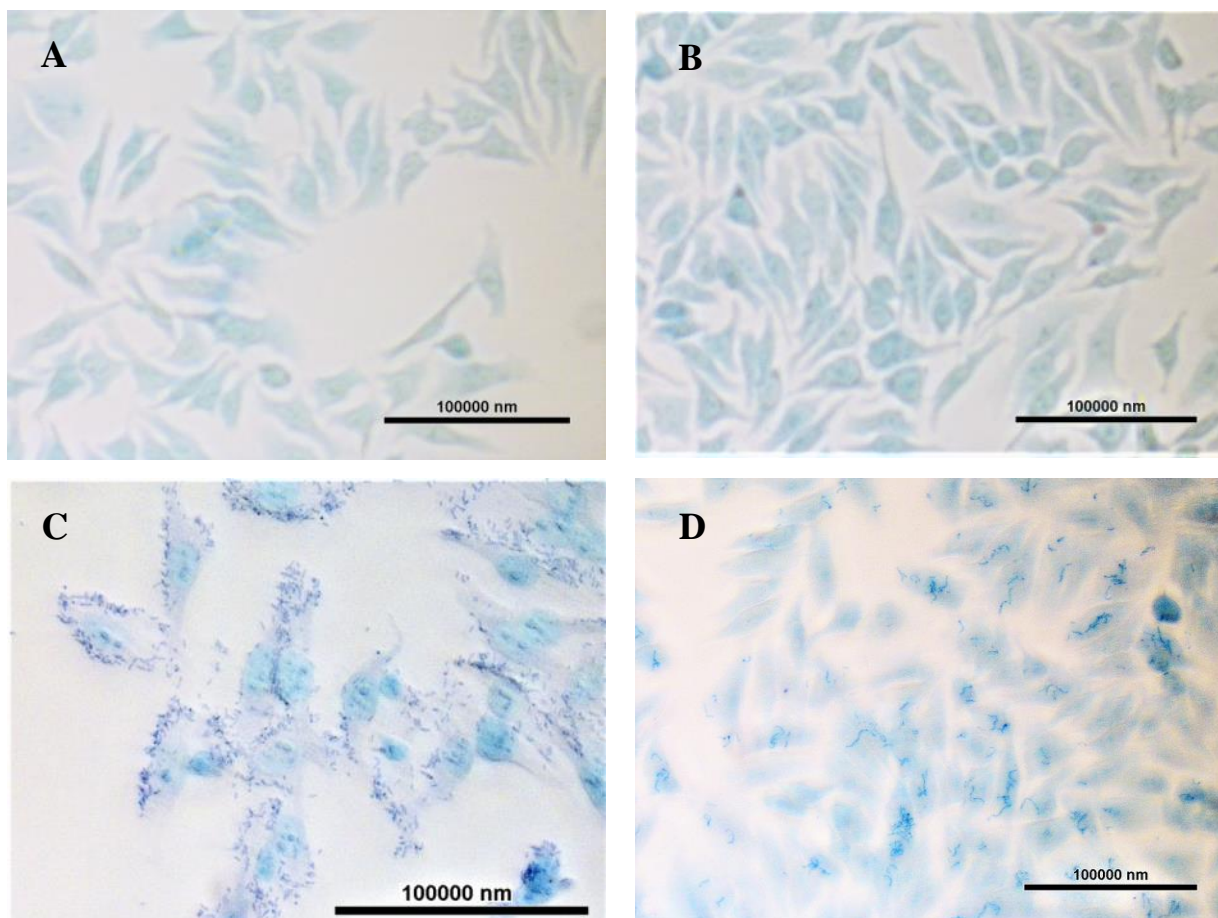


Figure 4.1. Microscopic images of HEp-2 cells, stained with safranin, methylene blue and trypan blue, $400\times$ magnification, scale bar – $100\text{ }\mu\text{m}$. A – cell culture with *C. rodentium*, B – cell culture with *E. coli* HB101 (control), C – cell culture with *L. rhamnosus* R0052, D – cell culture with *L. lactis* ATCC 11454.

4.5 Conclusions

Testing of our laboratory collection of LAB strains using *in vitro* antagonism tests and co-aggregation assay did not reveal a candidate having significant antagonistic effects on the growth of *C. rodentium*.

Since the attachment of *C. rodentium* to HEp-2 cells was not observed, it was not possible to test whether lactobacilli strains would affect the attachment.

However, *L. helveticus* R0052 and *L. lactis* ATCC 11454 showed a strong ability to attach to human cells. Based on these results and on previous research in the field it was decided to choose *L. helveticus* R0052 and *L. rhamnosus* R0011 strains that had been described by others as *Citrobacter* antagonists *in vivo* for future experiments using animal model of infectious colitis (Johnson-Henry *et al.*, 2005; Gareau *et al.*, 2010; Rodrigues *et al.*, 2012).

4.6 Preface to next chapter

Multiple lactobacilli strains were tested to select a bacterium able to inhibit the growth of *C. rodentium in vitro*. A strain *L. helveticus* R0052 showed a strong ability to attach to HEp-2 cells. Probiotics were chosen based on this result as well as on previous work done by others focusing on *C. rodentium* (Johnson-Henry *et al.*, 2005; Gareau *et al.*, 2010; Rodrigues *et al.*, 2012). Two lactobacilli strains – *L. rhamnosus* R0011 and *L. helveticus* R0052 – were selected for encapsulation to test the effects of PPI-alginate on the ability of these probiotic bacteria to inhibit pathogen-induced colitis in mice.

5 EFFICACY OF PEA-PROTEIN ALGINATE CAPSULES CONTAINING PROBIOTIC BACTERIA IN A MURINE MODEL OF *CITROBACTER RODENTIUM*-INDUCED COLITIS

5.1 Abstract

In the current study, I assessed the efficacy of two strains of *Lactobacillus* probiotics immobilized in a pea protein isolate - alginate microcapsules for efficacy using a mouse model of *Citrobacter rodentium*-induced colitis. The objective was to determine whether PPI-alginate encapsulation of a combination of *Lactobacillus rhamnosus* strain R0011 and *Lactobacillus helveticus* strain R0052 reduced the symptoms of colitis and/or caused any other non-target effects on the host. Accordingly, 4-week old mice were fed diets supplemented with freeze dried probiotics (group P), probiotic containing microcapsules (group PE) (lyophilized PPI-alginate microcapsules containing bacteria; 0.5 g of microcapsules per day; 10^8 CFU) or PPI-alginate microcapsules containing no bacteria (group E) (0.5 g per day). Half of the mice (controls, groups P, PE and E) were challenged by gavage with *C. rodentium* 2 weeks after the initiation of feeding. Daily monitoring of disease symptoms (abnormal behavior, diarrhea, etc.) and body weights were undertaken. At the termination of the study analyses of histopathological changes in colonic and cecal tissues, cytokine expression levels, pathogen and probiotic densities in fecal samples, as well as high throughput sequencing of mucosal microbial communities in the distal colon were also carried out. Infection of mice with *C. rodentium* led to marked progression of infectious colitis, which was evident from symptomatic and histopathological data and changes in cytokine expression.

Administration of the probiotics *L.rhamnosus* R0011 and *L. helveticus* R0052 led to changes in most of the disease markers. However, probiotics did not have a significant impact on altering cytokine profiles in infected animals. Based on cytokine expression analyses and histopathological data, it was evident that encapsulation materials (pea protein and calcium alginate) contributed to the development of inflammation and worsened a set of symptoms in the cecum. These results suggest that even though food ingredients may be generally recognized as safe, they

may in fact contribute to the development of an inflammatory response in certain animal disease models.

5.2 Introduction

The effective delivery of probiotics in sufficiently high numbers to the intestine for a beneficial effect is an ongoing challenge. When entering the human stomach, bacteria are exposed to adverse environmental conditions, such as low pH and pepsin. Ideally, a probiotic food product should contain protective substances or a technology that protects viable bacteria from these factors, allowing the bacteria to reach the colon in sufficient numbers in order to provide a functional or beneficial effect in the human or animal host. The most efficient methods for protection of bacteria from the harmful effects of acid and bile is their encapsulation within single or multiple biopolymer coatings, along with varying of biopolymer concentration and increasing of the initial cell loads (Chandramouli *et al.*, 2004; Petrović *et al.*, 2007; Annan *et al.*, 2008). The survival of bacteria is mainly related to the structural integrity of capsules, which involves various testing of different wall components to select favourable variants, such as microcapsules that are insoluble at acidic pH but dissolve under the more alkaline pH of the intestine (Narayani and Rao, 1995a,b; Nickerson *et al.*, 2006). One of the main criteria for success of a candidate encapsulation method is to promote the survival of bacteria in amounts equal to or greater than $7 \log \text{CFU/ml}^{-1}$ after being exposed to unfavorable conditions in stomach (Kotikalapudi *et al.*, 2009). Capsules also need to be non-toxic and pepsin-resistant.

With respect to the capsule wall composition, preference has been given to natural hydrocolloids and gums that are generally recognized as safe (GRAS) for human health. Compounds that have shown good protection of bacterial cells against acidic environment of the gut include alginate, carrageenans and gums (Holzapfel and Schillinger, 2002; Shah *et al.*, 2007; Bajaj *et al.*, 2007; Morris *et al.*, 2012). These biopolymers possess various useful features such as viscosity (thickening or gelling), water binding, emulsion stabilization, adhesion, foam stabilization and film formation (Chaplin, 2012). Due to these properties, it is easy to achieve various textures and forms by altering and combining physical factors. According to many probiotic encapsulation studies (Champagne and Fustier, 2007; Annan *et al.*, 2008; Chávarri *et al.*, 2010; Voo *et al.*, 2011), the most common material for immobilization of bacterial cells is alginate. This biopolymer is of algal origin and composed of (1 → 4)-linked β -d-mannuronic and α -l-guluronic acid residues (Voo *et al.*, 2011).

Water-holding capacity and solubility of alginate depends on various extrinsic factors such as pH, ionic strength and the origin of ions. For example, when sodium alginate is added to aqueous calcium chloride, it forms calcium alginate, a water-insoluble, gelatinous substance. This property of alginate is widely-used for the entrapment of probiotic bacteria. Bacterial cultures are added into an aqueous alginate solution, and in the presence of calcium chloride, leads to the formation of alginate microcapsules enmeshing the bacteria.

The use of pea protein isolate (PPI) in the food industry is gaining popularity because of its hypoallergenic properties and low concentration of isolate by-products (8-12 %) (Franco *et al.*, 2000; Tömösközi *et al.*, 2001). PPI is extracted from field peas (*Pisum sativum*) and contains two major globulin proteins: legumin (350-400 kDa) and vicilin (150 kDa) (Ducel *et al.*, 2004). Several studies have proven the advantages of using PPI in combination with sodium alginate during the formation of probiotic-containing microcapsules (Kotikalapudi *et al.*, 2010; Klemmer *et al.*, 2011), where PPI-alginate microcapsules showed enhanced protection of bacterial cells in simulated gastric juice. In order to form stable interactions with polyanions such as alginate, carrageenan or gellan gum, PPI should have an overall positive charge. Since the isoelectric point of PPI is pH ~ 4.5, favourable conditions for coacervation occur at pH < 4.5. On the contrary, at pH > pI, PPI is negatively-charged, and leads to segregative phase separation upon mixing with polyanions. In order to determine the proper conditions for formulation of stable interaction between protein and polysaccharide, the influence of various factors on complex formation needs to be evaluated (e.g., concentration, ratio, biopolymer-type, reactive groups present, molecular weight, pH, temperature, concentration of ions in the solution) (de Kruif *et al.*, 2004).

The efficacy of immobilized probiotics ultimately requires testing *in vivo*. In these cases, an animal model of a human disease is a valuable option. One such model, previously used for testing of probiotic properties of lactobacilli, is *Citrobacter rodentium*-induced colitis in mice (Johnson-Henry *et al.*, 2005; Rodrigues *et al.*, 2012). The infectious colitis caused by *C. rodentium* in mice is in many ways analogous to human disease caused by enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC, respectively) (Wong *et al.*, 2011; Crepin *et al.*, 2016). The infection of mice with 10⁸-10⁹ CFU of *C. rodentium* ATCC 51459 leads to infectious colitis characterized by colonic hyperplasia or elongation of the epithelial crypts, mononuclear immune cell infiltration, goblet cell depletion and other symptoms. The infection starts with the cecal patch, followed by progression to the distal colon 2-3 days post infection. In immunocompetent mouse

strains, the infection reaches its peak at day 7 post-infection (p.i.), and the clearance of the pathogen is achieved 3 to 4 weeks after infection (Collins *et al.*, 2014, 2016).

A mixture of *Lactobacillus* strains, *L. rhamnosus* R0011 and *L. helveticus* R0052, was used as a probiotic formulation. These two strains, mixed at a 95:5 ratio, have been patented and marketed as the probiotic Lacidofil™ and previously shown to reduce the severity of *C. rodentium* infection in mice (Johnson-Henry *et al.*, 2005; Gareau *et al.*, 2010; Rodrigues *et al.*, 2012).

Citrobacter rodentium as well as human pathogens EPEC and EHEC are collectively known as attaching and effacing (A/E) pathogens. They use a type III secretion system to inject effectors into enterocytes where they reprogram cell signaling (Wong *et al.*, 2011). Infection of mucosal surfaces by *C. rodentium* leads to the formation of attaching and effacing lesions. These lesions are characterized by effacement of the brush border microvilli and localized actin polymerization at the bacterial attachment site (Crepin *et al.*, 2016).

The infection of mice with *C. rodentium* causes colitis and a transmissible murine crypt hyperplasia (TMCH), characterized by intestinal dysbiosis: alterations of the structure, diversity and composition of both mucosa-associated and luminal microbiota (Hoffman *et al.*, 2009; Collins *et al.*, 2014). At the peak of the infection, *C. rodentium* numbers can reach 1 % - 3 % of the total bacterial counts shed in feces (Lupp *et al.*, 2007). The infection causes a reduction in obligate anaerobic bacteria, such as *Bacteroidaceae* and *Lachnospiraceae* families, and a loss of the genus *Ruminococcus* (Hoffman *et al.*, 2009). On the contrary the amounts of *Enterobacteriaceae* (of which *C. rodentium* is a member) increase, possibly due to ability of some bacteria of this family to metabolize reactive nitrogen and oxygen species in the inflamed intestine (Winter *et al.*, 2010; Winter *et al.*, 2013; Pham *et al.*, 2014).

The excessive induction of epithelial regeneration and repair mechanisms during TMCH leads to the development of colonic crypt hyperplasia, characterized by thickening of the colonic mucosa and marked elongation of the crypts (Schauer and Falkow, 1993). These repair mechanisms result in an accumulation of undifferentiated colonocytes at the luminal surface and a reduction in the number of goblet cells (Papapietro *et al.*, 2013). Colitis also causes the loss of epithelial barrier integrity and the transit of bacteria into the sterile lamina propria (Barker, van de Wetering and Clevers, 2008).

Clearance of *C. rodentium* is usually achieved within 2-3 weeks, when colonized epithelial cells are shed into the intestinal lumen, and the pathogen, opsonized by IgG antibodies directed

against virulence factors, is eliminated via phagocytosis by neutrophils and via the competing microbiota (Maaser *et al.*, 2004; Lebeis *et al.*, 2008; Kamada *et al.*, 2012).

According to previous studies administration of *L. rhamnosus* R0011 and *L. helveticus* R0052 to mice infected with *C. rodentium* improved survival of animals, ameliorated weight loss, colonic epithelial cell hyperplasia, mucosal barrier dysfunction (Johnson-Henry *et al.*, 2005; Gareau *et al.*, 2010; Rodrigues *et al.*, 2012). Pretreatment with probiotics reduced epithelial thickness, decreased bacterial internalization, restored goblet-cell formation, and increased mucus secretion (Johnson-Henry *et al.*, 2005). Probiotics also suppressed the fecal load of *C. rodentium* and the overgrowth of *Enterobacteriaceae* (Rodrigues *et al.*, 2012). Additionally a global decrease in diversity following *C. rodentium* infection was reported (Johnson-Henry *et al.*, 2005; Rodrigues *et al.*, 2012). Gareau *et al.* (2010) reported that in infected neonatal mice beneficial effects of probiotics were observed in B cell-deficient animals, indicating the requirement of T cells in reducing the adverse effects of colitis. In terms of cytokine expression levels, only a preliminary administration of probiotics (before infection with *C. rodentium*) was associated with suppression of *Ifn γ* and *Tnf α* transcripts 10 days after infection, while all other regimes, except for 6 days after infection, reduced *Il17* transcripts (Rodrigues *et al.*, 2012). In general a shift from a primarily pro-inflammatory T_h1 cell response to a more balanced T_h1/T_hr (regulatory) host immune response was observed when probiotic treatment of infected mice was implemented (Johnson-Henry *et al.*, 2005; Rodrigues *et al.*, 2012).

We have previously shown that pea-protein alginate microcapsules were able to protect acid-sensitive *Bifidobacterium adolescentis* *in vitro* and to deliver these bacteria to the lower parts of the rodent GIT (Varankovich *et al.*, 2015a). In this chapter, I tested whether *L. rhamnosus* R0011 and *L. helveticus* R0052 retain efficacy against *C. rodentium* induced colitis in mice, when encapsulated in the same type of matrix. Accordingly, the objective of the current study was to determine whether the PPI-alginate microcapsule delivery method of *L. rhamnosus* R0011 and *L. helveticus* R0052 interferes with their ability to reduce the symptoms of *C. rodentium*-induced colitis, or induces any other non-target effects on the host. It was hypothesized that probiotics would effectively inhibit the development of *C. rodentium*-induced colitis, and the use of PPI-alginate encapsulation would not cause any significant alterations in: a) microbiome composition of distal colon mucosa, b) expression levels of pro- and anti-inflammatory cytokines, c) disease symptoms, or d) cecal and colonic tissue inflammation and lesions, compared to non-encapsulated probiotics.

5.3 Materials and methods

5.3.1 Ethics statement

The study was carried out in strict accordance with the Canadian Council on Animal Care Guidelines. The project was approved by the Lethbridge Research and Development Centre (LRDC) Animal Care Committee (Animal Use Protocol 1511), and the LRDC Biosafety and Biosecurity Committee before commencement of the research.

5.3.2 Bacterial strains

Two bacterial strains, *L. rhamnosus* R0011 and *L. helveticus* R0052, provided by Lallemand (Lallemand Inc., Montreal, QC, Canada), were used in this study. These strains, mixed at a 95:5 ratio, have been patented as a probiotic mixture marketed under the name Lacidofil™ and have been previously shown to inhibit *C. rodentium* infection in C57BL/6 mice (Johnson-Henry *et al.*, 2005; Gareau *et al.*, 2010). Lyophilized cells were prepared as follows: strains were grown on MRS agar at 37°C for 48 h under anaerobic conditions. Isolated colonies were then separately seeded into MRS broth, incubated until the exponential phase (24 h and 36 h for strains R0011 and R0052, respectively), centrifuged at 5000 x g for 10 min, mixed with trehalose to a final concentration of 10 % and lyophilized (Labconco FreeZone 12 Free Dry System, Labconco Corp., Kansas, KS, USA). Freeze-dried cells of two strains were combined at a ratio of 95:5 and stored at 4°C. The number of living bacteria in the freeze-dried powder was monitored by plate counting on MRS every week.

5.3.3 Preparation of probiotic capsules

Capsule preparation was based on encapsulation and extrusion techniques described in Varankovich *et al.* (2015a). Pea protein isolate was kindly donated by Nutri-Pea Ltd. (Portage La Prairie, MB, Canada). Alginic acid sodium salt (medium viscosity; 61 % mannuronic and 39 % guluronic acid (M/G ratio of 1.56)), calcium chloride dehydrate and Tween 80 were purchased from Sigma-Aldrich (Oakville, ON, Canada). The composition of the wall material of microcapsules was as follows: 2 % PPI + 0.5 % (w/w) alginate. Concentration of PPI was adjusted to the protein content of the isolate (85 %); whereas, the polysaccharide concentration was used as is, on a per-weight basis. PPI was dissolved at 85°C and pH 9.0 for 2 h. Then, pH was adjusted to 7.0 and alginate was added to the mixture. After complete dissolution of the alginate (1-1.5 h at 85°C), the solution was cooled to room temperature (21-23°C) and mixed with 2 % (v/v) of the bacterial slurry, recovered by

centrifugation from 24 h bacterial broth (optical density at 600 nm was equal 1.305 (averaged; n=3)) (measured using Genesys 10S UV-Vis Spectrophotometer, Thermo Fisher Scientific, Madison, WI, USA). The final mixture (a blend of protein and alginate) was then extruded using a syringe and 18G (G, gauge) needle into 200 mL of ddH₂O containing Ca²⁺ ions (cross-linking agent) and Tween 80 (emulsifier). Prepared microcapsules were freeze-dried (Labconco FreeZone, Kansas City, MO, USA) for 15 h and stored at 4°C. The size of lyophilized microcapsules was ~ 2 mm in diameter. Capsules were ground to a size of ~ 0.5-1 mm to facilitate uptake by mice during feeding. The number of living probiotic bacteria in capsules was monitored by plate counting on MRS every week.

5.3.4 Animals and study design

The experiment was conducted as a four-by-two factorial design (i.e. probiotic/encapsulation × infection) arranged as a completely randomized design with four replicates. Every mouse was kept in a separate cage, and cages of the same treatment group were separated to make sure samples would be as independent as possible. Two replicates were conducted simultaneously, which was later accounted for in the statistical model as a random factor.

Specific pathogen-free (SPF) female C57BL/6 mice (n = 32, 3-week old) were purchased from Charles River Laboratories (Montreal, QC, Canada) and allowed to acclimatize to the Lethbridge Research Center Vivarium for 7 days before commencement of the experiment. Mice were fed with freeze-dried probiotic cells (group P), probiotic microcapsules (group PE) (lyophilized PPI-alginate microcapsules containing bacteria; 0.5 g of microcapsules per day; 10⁸ CFU) or blank PPI-alginate microcapsules (group E) (0.5 g per day), starting at four weeks of age. Half of the control mice, and half from P, PE and E groups, were gavaged with *C. rodentium* 2 weeks after the start of feeding (groups C, PC, PEC and EC, respectively). Sterile rodent chow (basal AIN-93G diet; Harlan laboratories, Madison, WI, USA) and water were available at all times. Capsules were first ground then mixed with 5 g of jello (“Jell-o” brand; contents: sugar, gelatin, adipic acid, disodium phosphate, sodium citrate, fumaric acid, red 40) to ease the feeding process. Control mice were fed 5 g of plain jelly per day. AIN-93G-VX vitamin mix (94047; 10 %, w/v) and AIN-93G-MX mineral mix (94046; 15 %, w/v) (Harlan laboratories, Madison, WI, USA) were dissolved in the jelly.

5.3.5 Infection of mice with *Citrobacter rodentium*

Initially, *C. rodentium* Schauer *et al.* (ATCC[®] 51459[™]; laboratory collection of Dr. G. Douglas Inglis, Lethbridge Research Centre) was plated from frozen glycerol stock onto LB agar and incubated for 24 h at 37°C. Then, 150 µl of sterile LB broth was applied to a plate of *C. rodentium*. Cells from ¼ of plate were gently suspended in broth and transferred to a flask with 150 ml of sterile LB. Broth was then incubated on a shaker (110 rpm) for approximately 2 h until the absorbance reached approximately 0.1 at OD₆₀₀. Cells were centrifuged at 4000 x g for 15 min and then resuspended in 3 mL of 1X sterile PBS. Each mouse was gavaged with 100 µl of the *C. rodentium* solution using a bulb-tipped gastric gavage needle attached to a 1 ml syringe. The dosage (10⁸ CFU) was confirmed by plating on LB agar.

5.3.6 Sample collection

Body weight, animal survival, and behavior were monitored daily throughout the experimental period. Fresh fecal pellets were collected every second day during four weeks of the experiment. At the completion of the experiment 10 days post-infection, mice were anesthetized with isoflurane and euthanized via cervical dislocation. Immediately after death, a mid-line laparotomy was used to exteriorize the intestine, and a gross pathological assessment of the intestine and associated tissues was completed. The intestine, liver and mesenteric lymph nodes were harvested aseptically. Several distal colon and cecum segments were either weighed and frozen at -80°C for DNA extraction and analysis of bacterial communities, or fixed in 10 % neutral-buffered formalin for histopathologic analyses. Samples of distal colon were also placed in RNAlater (Qiagen Inc., Toronto, ON) and stored at -20°C for mRNA extraction for cytokine analysis.

5.3.7 Histopathological analysis

Colonic and cecal tissue samples were fixed in 10 % buffered formalin for 24 h, dehydrated, embedded in paraffin, sectioned, de-paraffinized with xylene, and stained with hematoxylin and eosin (Jiminez *et al.*, 2016). Epithelial cell hyperplasia, crypt height, epithelial injury, inflammation, goblet cell depletion, mitotic activity and fibrosis in tissues were assessed by a veterinary pathologist (Richard R. E. Uwiera) in a double-blind fashion using a scoring criteria described previously (Costa *et al.*, 2011). In brief, tissue sections were graded from 0 to 4 for epithelial cell hyperplasia, crypt height, epithelial injury, inflammation, and 0 to 3 for mitotic activity of epithelial cells and goblet

cell depletion. A total pathology score was obtained by calculating the sum of scores for all categories for each mouse.

5.3.8 Cytokine expression

Changes in the expression of the cytokines of pro-inflammatory- (*Il17*, *Infγ*) and anti-inflammatory (*Il4*, *Tgfβ*) profiles were determined.

Total RNA from samples of distal colon tissue was isolated using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was then reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Expression differences of cytokine genes between groups were analyzed by real-time PCR using QuantiTect SYBR Green MasterMix (Qiagen, Valencia, CA, USA). The reaction master mix was prepared as follows: QuantiTect SYBR MM – 10 µl, primers – 1 µl each, bovine serum albumin (BSA; Promega Inc., Madison, WI, USA) – 2 µl, RNase free water – 4 µl. Template volume – 2 µl. Total reaction volume – 20 µl. Cycling conditions: 95°C for 15 min, followed by 40 cycles of 94°C – 15 s, 58°C – 30 s, and 72°C – 30 s. Negative extraction controls, as well as non-template controls, were added to the plate in every qPCR run. Melting curve analysis was performed as an additional quality control. Only one peak for each qPCR product was present on the curve.

Samples were run in triplicate on a Bio-Rad CFX Connect thermocycler (Bio-Rad Laboratories Inc., Mississauga, ON, Canada). Primer sequences are listed in Table 5.1. Beta-glucuronidase (*GusB*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*) and peptidylpropyl isomerase A (*Ppia*) were used as endogenous controls. *Hprt* and *Ppia* were selected as the optimal set of normalization genes using NormFinder software (Andersen *et al.*, 2004). Results were analyzed using the relative quantification ($\Delta\Delta C_t$) method and expressed as fold change \pm SEM. The qPCR efficiency for every target and reference gene was calculated according Liu and Saint (2002). Relative Expression Software Tool (REST, version 2009; Pfaffl and Qiagen, Hilden, Germany) was used to perform calculations.

Table 5.1. Primers for qPCR.

| Name | Sequence (5'-3') | Annealing temperature | Source |
|---------------------|--------------------------------|-----------------------|------------------------------|
| <i>mGusB-f</i> | GCT CAT CTG GAA TTT CGC CG | 58°C | Brown <i>et al.</i> , 2016 |
| <i>mGusB-r</i> | CGG TTT CGT TGG CAA TCC TC | 58°C | |
| <i>Ppia-f</i> | CTA AAG CAT ACA GGT CCT GGC A | 58°C | |
| <i>Ppia-r</i> | ATG CCT TCT TTC ACC TTC CCA | 58°C | |
| <i>Hprt-f</i> | ACA GGC CAG ACT TTG TTG GAT | 58°C | |
| <i>Hprt-r</i> | ACT TGC GCT CAT CTT AGG CT | 58°C | |
| <i>mIL17a-f</i> | GCA GCG ATC ATC CCT CAA AG | 58°C | |
| <i>mIL17a-r</i> | ACG TGG AAC GGT TGA GGT AG | 58°C | |
| <i>mIfng-f</i> | ACG GCA CAG TCA TTG AAA GC | 58°C | |
| <i>mIfng-r</i> | TCT GGC TCT GCA GGA TTT TCA | 58°C | |
| <i>murTGFBeta-f</i> | TGC TGC TTT CTC CCT CAA CCT | 58°C | |
| <i>murTGFBeta-r</i> | CAC TGC TTC CCG AAT GTC TGA | 58°C | |
| <i>IL4-f</i> | CAT CGG CAT TTT GAA CGA GGT CA | 58°C | |
| <i>IL4-r</i> | CTT ATC GAT GAA TCA GGC ATC G | 58°C | |
| <i>CrodF</i> | GCTTCTGCGAAGTCTGTCAA | 58°C | Jiminez <i>et al.</i> , 2016 |
| <i>CrodR</i> | CAGTAAAGCGACTTAACAGATT | 58°C | |
| <i>R0011-f</i> | ACTCCAAAGAGCATTACCTCCG | 60°C | Lallemand Health Solutions |
| <i>R0011-r</i> | TGAATATGCCGATCTAAGTCCA | 60°C | |
| <i>R0052-f</i> | AGAATCAAGCAGAGACTGGCTACG | 60°C | |
| <i>R0052-r</i> | GGACCGGATTTGAGTAGAGGTA | 60°C | |

5.3.9 Quantification of pathogen and probiotics in mice fecal samples

Extraction of DNA from murine fecal samples was carried out with QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions. Primer sequences for *L. rhamnosus* R0011 and *L. helveticus* R0052 were provided by Lallemand (Lallemand Inc.). The primer pair for strain R0011 is specific to phage 113A29, which is unique to this strain; plasmid pIR52 was used to design a primer for R0052 (plasmid strain-specific for *L. helveticus* R0052) (Table 5.1). Primers used for qPCR-based quantification of *C. rodentium* were *CrodF* and *CrodR* (Table 5.1).

All samples were run in triplicate on a Bio-Rad CFX Connect thermocycler (Bio-Rad Laboratories Inc., Mississauga, ON, Canada). Master mix was prepared as follows: QuantiTect SYBR MM – 10 µl, primers – 1 µl each, bovine serum albumin (BSA; Promega Inc., Madison, WI, USA) – 2 µl, RNase free water – 4 µl. Template volume – 2 µl. Total reaction volume – 20 µl. Cycling conditions were: 95°C for 15 min, followed by 40 cycles at 94°C – 15 s, 60°C (58°C in the case of *CrodF/R* primers) – 30 s, 72°C – 30 s. Negative extraction controls, as well as non-template

controls, were added to the plate in every qPCR run. Dilutions of DNA, extracted from pure cultures of *C. rodentium*, *L. rhamnosus* R0011 and *L. helveticus* R0052 and quantified fluorometrically (Qubit, Life Technologies, Burlington, ON, Canada), were used to construct standard curves (10^6 to 10^1 copies; $n = 2$) for each run. Primer efficiency was between 85–105 % and a single peak for each target gene was present on a melting curve.

5.3.10 High-throughput amplicon sequencing

5.3.10.1 Library preparation and sequencing

Phylogenetic profiles of the mouse mucosa-associated microbiome of distal colon tissue were generated by performing massively-parallel sequencing of the 16S rRNA gene on an Illumina MiSeq system (Illumina Inc., San Diego, CA, USA) using the V3 MiSeq Reagent kit (600 cycles; Illumina Inc.). DNA was extracted from equally-sized pieces of distal colon samples (two pieces, ~ 15 mg each) with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The primers used to target the 16S V3 and V4 regions of the gene were *S-D-Bact-0341-b-S-17* (forward) and *S-D-Bact-0785-a-A-21* (reverse) (expected sequence length – 428 pb) (Klindworth *et al.*, 2013). The library preparation step of the MiSeq workflow was based on 16S Metagenomic Sequencing Library Preparation guide (Illumina Inc.) with modifications: the first out of two PCR clean-ups was done through gel extraction/purification (0.7 % agarose (w/v), 110V, 500A, 45 min); Qubit (Life TechnologiesTM Corp., Carlsbad, CA, USA; HS DNA assay) was used instead of a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA); PhiX was added to final pooled libraries at a concentration of 10 %. Libraries were normalized to 4 nM in 6 ml volumes each. The loading concentration was 8 pM. Loading of the libraries onto the instrument was performed according to the MiSeq Reagent Kit V3 Reagent Preparation Guide (Illumina Inc.).

5.3.10.2 Analysis of 16S rRNA genes

De-multiplexing was performed using MiSeq Reporter software (Illumina Inc.) allowing for a single mismatch in index recognition. Sequencing data were processed using QIIME (Quantitative Insights Into Microbial Ecology; Caporaso *et al.*, 2010). To classify experimental data, de-multiplexed reads were merged in QIIME, using FLASH (Magoc and Salzberg, 2011) with minimum and maximum overlap settings of 20 and 200 bp, respectively. Reads were then pooled together and filtered using default settings with *multiple_split_libraries_fastq.py*. OTU assignment

was performed with *parallel_pick_otus_uclust_ref.py* using Silva database v. 123 and the open-reference method (Edgar, 2010; Yilmaz *et al.*, 2013).

5.3.10.3 Taxonomic diversity analysis and statistics

A rarefaction depth of 16000 was chosen based on the lowest number of sequences among the 31 samples (one of the samples was excluded due to poor quality). The core microbiome at Phylum and Family levels was calculated with *compute_core_microbiome.py*. For alpha diversity statistics Chao1, shannon and simpson metrics were calculated and compared (Student's t-test, p -value was set to ≤ 0.05) using *alpha_diversity.py*, *collate_alpha.py* and *compare_alpha_diversity.py* in QIIME. Unweighted UniFrac was chosen for calculation of beta diversity metrics (Lozupone and Knight, 2005). Dissimilarity matrix calculations and principal coordinate analyses were performed using jackknifing at the rarefaction depth of 16000 (*jackknifed_beta_diversity.py*), and analyzed using *dissimilarity_mtx_stats.py* and *make_distance_boxplots.py*, which calculates two-sample t-tests for all pairs of boxplots with Bonferroni-corrected p -values for each test (Vazquez-Baeza *et al.*, 2013). Statistics for histology scoring and cytokine expression was performed in SPSS Statistics v. 23.0 (IBM Corp., Armonk, NY, USA; 2015) (level of significance for every test used was set to ≤ 0.05).

5.3.11 Statistics

Continuous data (weight gain, food intake, etc.) was checked for normality and tested using MIXED procedure in SPSS v. 23.0 (IBM Corp.) with replicate structure factor (1, 2 versus 3, 4) treated as a random effect. The lowest Akaike's Information Criterion was used in each case to choose a best-fitted model with an appropriate covariance structure. Relative expression of cytokines was calculated using the pair-wise fixed reallocation randomization test (PWFR test) in REST[®] (5000 randomizations) (Pfaffl *et al.*, 2002). Fisher's Exact Test and a Two-way ANOVA on ranks (for interactions analyses) in SPSS were used to analyze histology scores. Pathogen and probiotic qPCR quantification results were analyzed by a One-way ANOVA with repeated measures in SPSS. Probability values of ≤ 0.050 were considered significant. Correlations were performed using Spearman's rank correlation method in SPSS. Corrections for multiple comparisons were performed using Bonferroni (high throughput sequencing analyses) or Sidak (in all other cases) methods.

Graphs were constructed in GraphPad Prism (Version 7.02; GraphPad Software Inc., San Diego, CA, USA).

5.4 Results

5.4.1 Symptoms and necropsy data

Infection of mice with *C. rodentium* ATCC 51459 can result in severe weight loss and 100 % mortality ten days post-infection (Rodrigues *et al.*, 2012); however, this effect was not always observed in C57BL/6 mice (Johnson-Henry *et al.*, 2015). In the present study, none of the animals showed any abnormal behavior or overt disease symptoms, such as diarrhea. There was no significant effect of any single treatment type (PEC, PE, PC, P, C, E or EC) on loss of weight in mice (Fig. 5.1A); however, and in general, non-infected mice gained more weight by the end of the experiment compared to infected mice ($F = 6.796$, $p = 0.014$). Necropsies of infected mice revealed inflammation of the colon ($p < 0.001$; compared to non-infected groups; data not shown), enlarged spleens ($p = 0.052$ for spleen weight (Fig. 5.1C), but $F = 4.691$ at $p = 0.039$ for spleen length (Fig. 5.1D)) and notably enlarged lymph nodes ($p = 0.056$; Fig. 5.1B). Food intake was also slightly increased in non-infected mice, though the effect was not significant (data not shown). The average feed intake per mouse per day was 2.68 ± 0.22 g. Treatment type did not affect ($F = 1.668$, $p = 0.167$) the length of the colon (data not shown), however, infected mice had ($F = 4.913$, $p = 0.037$, mean difference = 0.342 mm) shorter colons than non-infected. Liver weights did not differ between infected and non-infected mice, or between treatment groups.

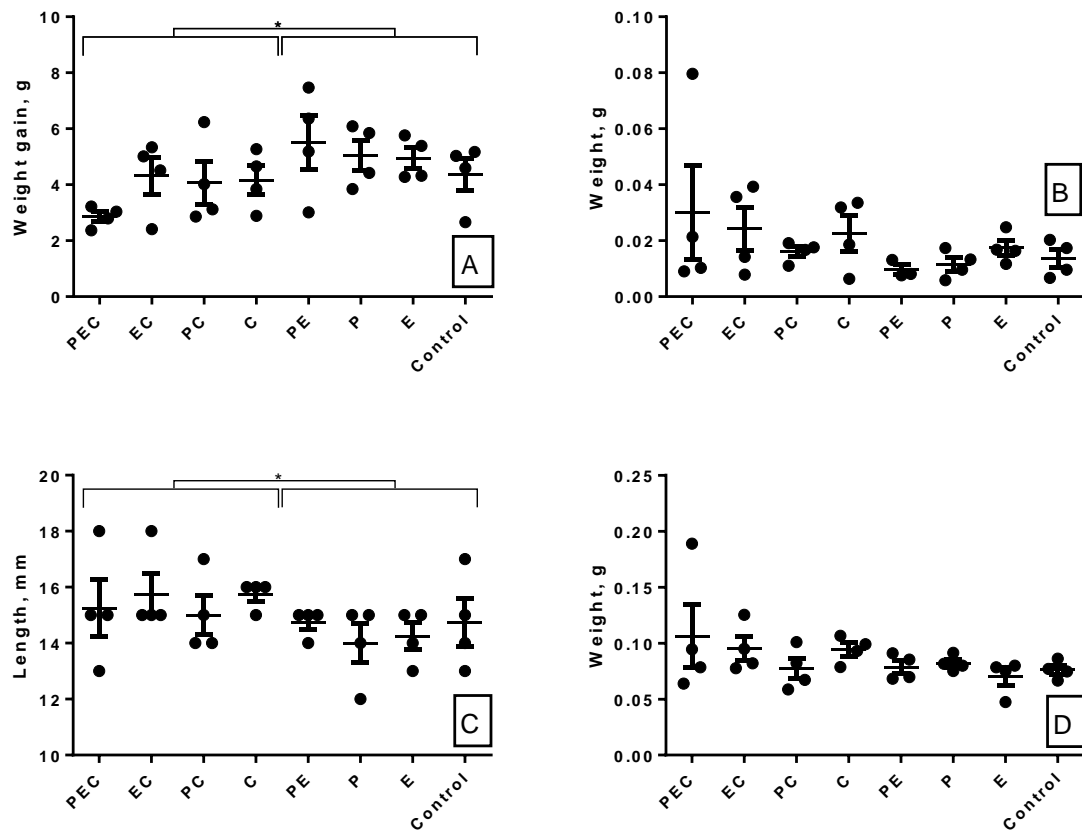


Figure 5.1. Effect of treatment on the weight gain and necropsy data of mice, challenged with *C. rodentium*, and/or treated with probiotics. A – weight gain, B – lymph node weight, C – spleen length, D – spleen weight. Data represents mean \pm standard error of the mean (SEM) and an asterisk represents the probability value $p \leq 0.050$ ($n = 4$) as determined by F-test (Mixed ANOVA). Group abbreviations key: P – probiotics, C – *C. rodentium* infection, E – encapsulation.

5.4.2 Histopathology

Histopathological analysis confirmed the effect of *C. rodentium* infection on the development of epithelial hyperplasia and injury, fibrosis, mitotic activity and other symptoms in both the colon and cecum (data not shown). The impact of infection differed considerably for those two segments of the gut. A worsening of epithelial hyperplasia was observed in infected vs. non-infected mice in the colon ($F = 10.878$, $p = 0.008$), with an interaction between infection and probiotic in cecum ($F = 7.604$, $p = 0.001$). Infected mice that received probiotics (PC) had lower histopathological scores than either the control group or mice fed probiotics (P; data not shown). A similar interaction between probiotic and infection was observed in the cecum for goblet cell depletion ($F = 9.000$, $p = 0.006$), inflammation ($F = 20.557$, $p < 0.001$), epithelial injury ($F = 5.064$, $p = 0.034$) and mitotic activity ($F = 6.313$, $p = 0.019$). In all these cases a corresponding symptom was less prominent with the addition of probiotic.

Interestingly, EC treatment (blank microcapsules + infection) ($p < 0.05$) worsened symptoms in the cecum, including epithelial hyperplasia, increased crypt height (also in colon), inflammation, mitotic activity and goblet cell depletion – compared to some of the other treatments (data not shown). In the colon, pathogen challenge had a negative impact ($p < 0.05$) on all the observed symptoms, with the exception of fibrosis.

Average total histopathological scores for colon were higher ($F = 14.517$, $p = 0.001$) in infected compared to non-infected mice, whereas the total score for the cecum was mediated by the probiotic-infection interaction ($F = 11.680$, $p = 0.02$); infected mice receiving probiotics had lower total histopathological scores compared to mice not fed probiotics (Fig. 5.2). In general, and as expected, the most prominent effect of *C. rodentium* – induced colitis was observed in infected groups of mice, with probiotics mediating the development of the disease in cecum. One complicating factor was the development of mild colitis symptoms (e.g., inflammation) in control mice as well as in other non-challenged treatment groups.

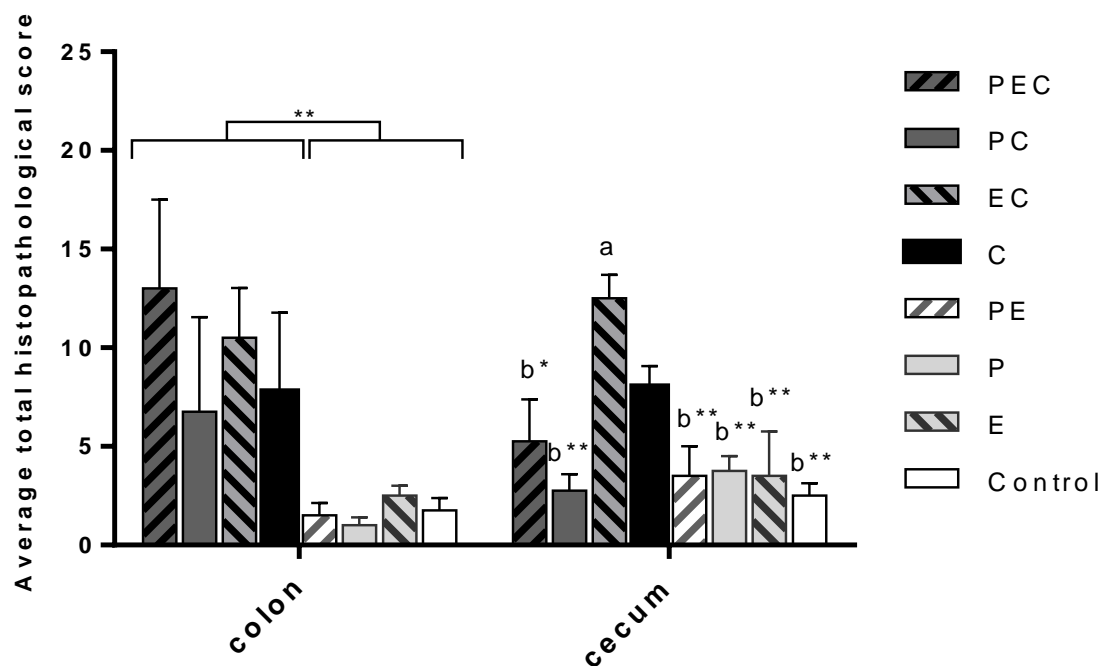


Figure 5.2. Effect of treatment on average total histopathological scores in the colon and cecum of mice challenged with *C. rodentium*. Data represents an average (mean \pm SEM) of total scores for all histopathological markers of each mouse in a treatment group ($n = 4$) and asterisks represent the probability value $*p \leq 0.050$ and $**p \leq 0.010$ (F-test in Mixed ANOVA). Bars marked with different letters are significantly different from each other. Group abbreviations key: P – probiotics, C – *C. rodentium* infection, E – encapsulation.

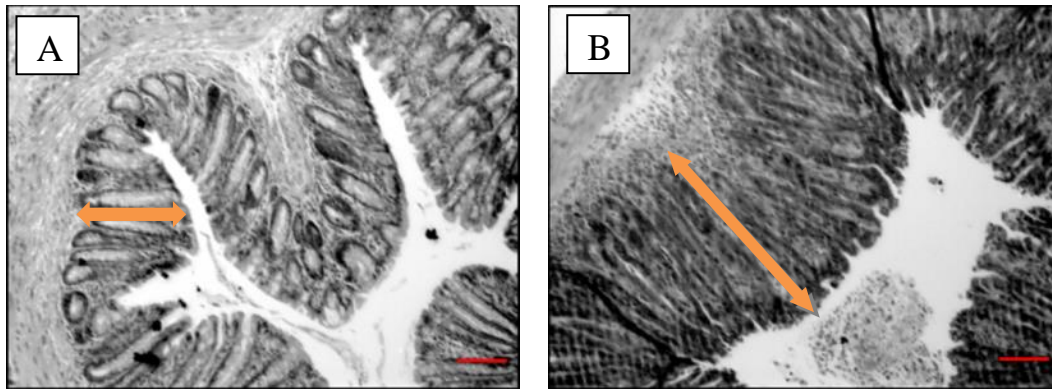


Figure 5.3. Histomicrographs of the mouse colon. A – control mouse, B – mouse infected with *C. rodentium*. Prominent crypt extension and epithelial hyperplasia were evident in mice treated with *C. rodentium*. Arrows indicate the difference in crypt height. Scale bar = 100 μ m.

5.4.3 Cytokine expression

Concentrations of T_H1 - (*Inf* γ), T_H2 - (*Il4*), T_H17 - (*Il17*), and T_{reg} - (*Tgf* β) cytokines in tissues of distal colon were quantified by qPCR (Fig. 5.4). *Il17* was expressed at higher levels in the distal colon tissues of infected compared to non-infected mice ($F = 7.559$, $p = 0.012$) (Fig. 5.4), and was up-regulated (in comparison to the control group) in the PEC group by a mean factor of 43.708 (PWFR test, $p = 0.049$; Fig. 5.4).

Inf γ was up-regulated in the EC group by a mean factor of 4.401 (PWFR test, $p = 0.035$), and in the PEC group by a mean factor of 12.688 (PWFR test, $p = 0.042$) in comparison to the control group (Fig. 5.4). No effects from infection, probiotic or encapsulation were found on concentrations of *Inf* γ mRNA. In general, infected animals had three times-higher relative expression levels of this cytokine than did non-infected animals (4.179 (SE = 1.212) vs. 1.366 (SE = 1.212), $p = 0.058$). In the PEC group, probiotics were not able to suppress the expression of the pro-inflammatory cytokine.

Il4 was up-regulated in the EC group (in comparison to control group) by a mean factor of 4.235 (PWFR test, $p = 0.005$; Fig. 5.4). Infected mice were found to have higher concentration of *Il4* mRNA in distal colon tissue than did non-infected mice ($F = 8.246$, $p = 0.010$). In general, neither encapsulation material nor probiotic administration affected *Il4* mRNA expression.

There was no difference in expression levels of transforming growth factor beta (*Tgf-β1*) between treatments. Though not considered significant, there was an approximate two-fold increase in relative concentration of mRNA of this cytokine in infected animals compared to non-infected animals (2.954 (SE = 0.522) vs. 1.410 (SE = 0.579); $p = 0.061$) (Fig. 5.4).

5.4.4 Pathogen and probiotic densities in mice fecal samples

Pathogen and each probiotic were quantified by qPCR in fecal samples of mice taken at three time points – before probiotic administration, before *C. rodentium* inoculation (before infection), and before necropsy (Fig. 5.5). No pathogen was detected in fecal samples collected prior to initiation of the experiment (data not shown). Prior to necropsy, *C. rodentium* was present in feces at a concentration of ~ 7-7.5 log CFU/g, and there was no difference in numbers of the pathogen between treatments (Fig. 5.5A).

L. rhamnosus R0011 was present in treated mice at concentrations of ~ 6.5-7 log CFU/g of feces up to the final sampling period (Fig. 5.5B). Additionally, at the end of the experiment, the PE group (probiotic capsules) had significantly higher densities of strain R0011 compared to the P group (non-encapsulated probiotic), which suggests that encapsulation of probiotics increased their numbers in fecal samples ($F = 4.698$, $p = 0.006$). Interestingly, numbers of R0011 decreased slightly (by ~ 0.5 log CFU/g in each group; $F = 49.386$, $p < 0.001$; Fig. 5.5B). The second strain, *L. helveticus* R0052, was also present in feces of all mice treated with probiotics at a concentration of ~ 5-6 log CFU/g (Fig. 5.5B). No difference in its numbers was found between treatments. However, a downward trend in CFU/g for this probiotic was observed by the end of the experiment, but only for challenged groups (by less than 0.5 log CFU/g; $F = 6.577$, $p = 0.015$). Primers for qPCR used in these experiments were targeted the small cryptic plasmid, pIR52, specific to strain R0052, and the gene for the 113A29 phage head protein, specific for strain R0011 (Hagen *et al.*, 2010; Lallemand Inc., unpublished data).

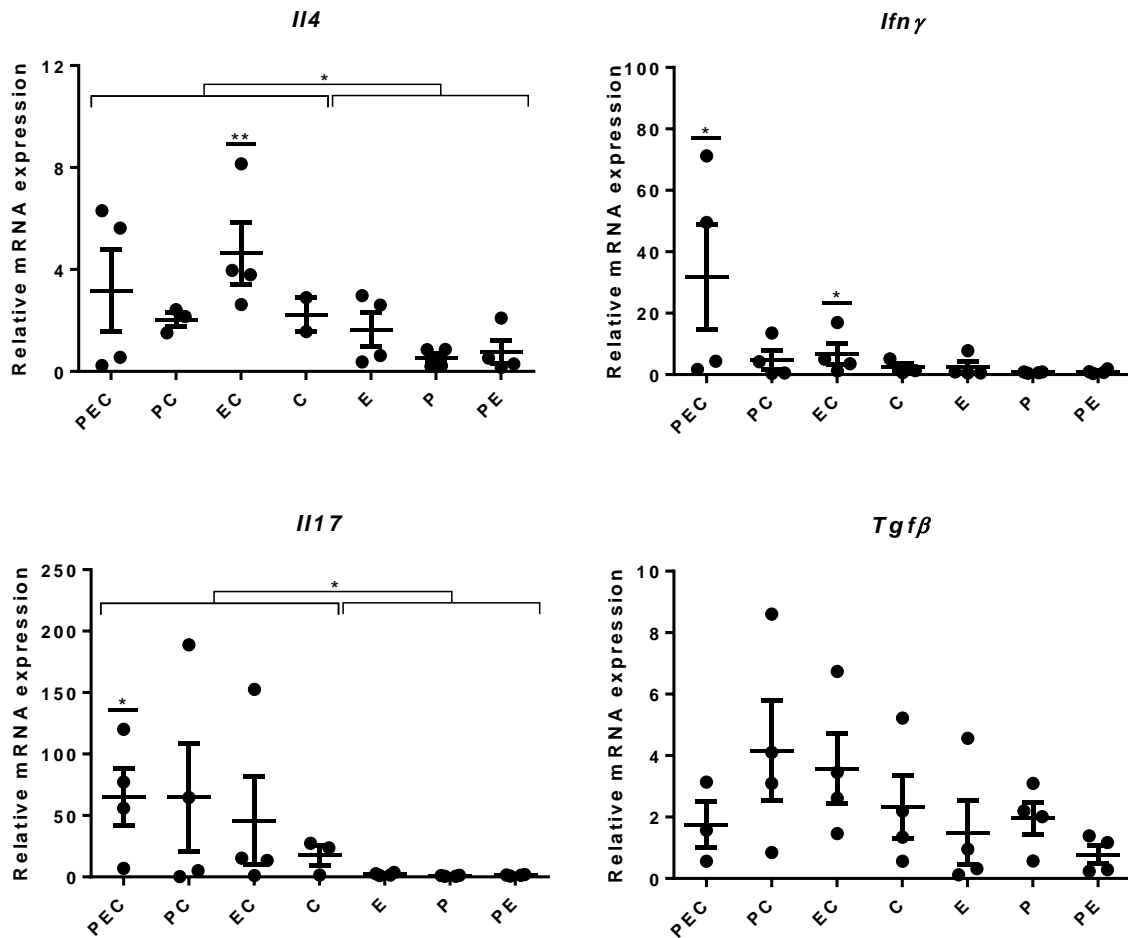


Figure 5.4. Relative cytokine expression in the distal colon of mice challenged with *C. rodentium* and/or treated with encapsulated or non-encapsulated probiotics in comparison with the control group as measured using qPCR (*Ppia* and *Hprt* as reference targets). Data is presented as fold change \pm SEM (n = 4; 2 in case of *IL4*/C group) and asterisks represent the probability value $*p \leq 0.050$ (F-test in Mixed ANOVA). Group abbreviations key: P – probiotics, C – *C. rodentium* infection, E – encapsulation.

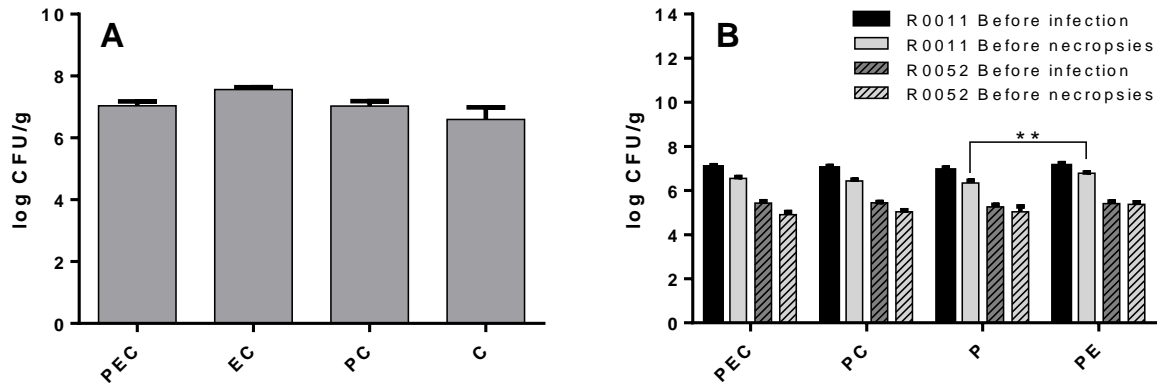


Figure 5.5. *C. rodentium* (A) and probiotic (B) counts in fecal samples from mice challenged with this pathogen or treated with encapsulated or non-encapsulated probiotics. A – *C. rodentium* counts before necropsy, B – *L. rhamnosus* R0011 and *L. helveticus* R0052 concentrations before infection and before necropsy. Data is presented as log CFU/g \pm SEM (n = 4) and asterisks represent the probability value $**p \leq 0.010$ (F-test in repeated measures ANOVA). Group abbreviations key: P – probiotics, C – *C. rodentium* infection, E – encapsulation.

5.4.5 16S rRNA gene analysis

The mucosa-associated microbial communities in the distal colon were characterized by 16S rRNA gene analysis. One sample (PEC treatment group) was excluded from all downstream analyses due to poor quality. Sequences were assigned to the phylogeny using SILVA database. The sequences were assigned to 5-14 different OTUs at the Phylum level and from 21 to 66 OTUs at the Family level – (Table 5.2).

Chao1, shannon and simpson metrics were used to assess alpha diversity of samples. Though control mice and animals fed with blank microcapsules had the highest richness and diversity in mucosa-associated microbial composition, there was no detectable difference in total species richness (Chao1) or diversity (shannon, simpson) within samples of different treatment groups (Table 5.2).

Table 5.2. Number of OTUs and alpha diversity metrics. Abbreviations key: P – probiotics, C – *C. rodentium* infection, E – encapsulation.

| Sample | Number of OTUs at the Phylum level | Number of OTUs at the Family level | chao1 | shannon (min-max) | simpson (min-max) |
|----------|--|---------------------------------------|-------------|----------------------|----------------------|
| Control1 | 13 | 56 | 2869 ± 2632 | 6.907-8.613 | 0.968-0.973 |
| Control2 | 12 | 49 | 4403 ± 4158 | 7.243-9.22 | 0.973-0.974 |
| Control3 | 11 | 60 | 4744 ± 5629 | 6.824-8.204 | 0.977-0.979 |
| Control4 | 12 | 57 | 4105 ± 5131 | 6.793-7.803 | 0.982-0.983 |
| C1 | 9 | 41 | 4057 ± 5104 | 6.667-7.744 | 0.979-0.98 |
| C2 | 7 | 23 | 1322 ± 1749 | 2.616-2.748 | 0.745-0.744 |
| C3 | 10 | 48 | 4937 ± 6045 | 7.323-8.497 | 0.989-0.991 |
| C4 | 5 | 21 | 962 ± 1266 | 3.11-3.246 | 0.818-0.812 |
| E1 | 10 | 43 | 5026 ± 6243 | 6.981-8.292 | 0.986-0.989 |
| E2 | 8 | 32 | 1846 ± 1691 | 6.696-7.929 | 0.96-0.963 |
| E3 | 9 | 44 | 4803 ± 6054 | 7.061-8.301 | 0.987-0.989 |
| E4 | 11 | 40 | 2918 ± 3525 | 6.786-7.687 | 0.984-0.986 |
| EC1 | 9 | 39 | 1679 ± 2097 | 2.667-3.075 | 0.747-0.762 |
| EC2 | 8 | 38 | 2670 ± 2221 | 6.281-7.807 | 0.945-0.943 |
| EC3 | 11 | 56 | 3595 ± 4577 | 5.134-5.91 | 0.923-0.925 |
| EC4 | 9 | 35 | 1390 ± 1849 | 2.701-2.933 | 0.762-0.767 |
| P1 | 14 | 66 | 3290 ± 3454 | 7.068-8.611 | 0.98-0.982 |
| P2 | 9 | 39 | 2900 ± 2254 | 7.027-8.713 | 0.968-0.97 |
| P3 | 11 | 53 | 1808 ± 2381 | 3.244-3.418 | 0.805-0.792 |
| P4 | 10 | 43 | 2467 ± 2585 | 6.653-7.643 | 0.981-0.983 |
| PC1 | 6 | 27 | 1383 ± 1834 | 2.603-2.927 | 0.748-0.757 |
| PC2 | 10 | 37 | 2631 ± 3087 | 4.947-5.789 | 0.899-0.908 |
| PC3 | 9 | 48 | 4997 ± 6265 | 7.12-8.244 | 0.987-0.988 |
| PC4 | 11 | 47 | 2462 ± 2582 | 5.697-6.825 | 0.93-0.932 |
| PE1 | 10 | 42 | 3818 ± 4765 | 7.028-8.183 | 0.988-0.991 |
| PE2 | 9 | 32 | 1115 ± 1294 | 4.457-4.992 | 0.887-0.892 |
| PE3 | 11 | 41 | 2030 ± 2536 | 4.017-4.442 | 0.875-0.872 |
| PE4 | 9 | 40 | 3240 ± 3890 | 6.465-7.43 | 0.973-0.975 |
| PEC2 | 8 | 28 | 1027 ± 1222 | 2.71-3.029 | 0.748-0.762 |
| PEC3 | 9 | 42 | 1684 ± 2166 | 3.035-3.272 | 0.773-0.773 |
| PEC4 | 9 | 33 | 1499 ± 1801 | 3.725-4.04 | 0.829-0.821 |

5.4.5.1 Taxonomy

At the phylum level samples were primarily composed of OTUs aligning within the *Bacteroidetes*, *Defferibacteres*, *Firmicutes* and *Proteobacteria*, all of which are typically occurring taxa for the murine gut (Figs. 5.6, A.1). At the family level, *Enterobacteriaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidaceae*, *Defferibacteraceae* and *Lactobacillaceae* were present in all the samples (Figs. 5.8, A.2).

In general, mucosa-associated communities of control mice were dominated by *Firmicutes* (*Lachnospiraceae*, *Ruminococcaceae* and *Lactobacillaceae*) (Figs. 5.6, A.1, 5.7, A.2). Infection with *C. rodentium* caused increase in *Proteobacteria* (*Enterobacteriaceae*) up to 95% of the recovered OTUs in two out of four animals, while the microbiome of the other two mice was dominated by *Firmicutes* (*Lachnospiraceae* and *Ruminococcaceae*) (Figs. 5.6, A.1, 5.7, A.2). The administration of probiotics to infected mice (PC) led to the decrease in *Firmicutes* (*Lachnospiraceae*) and increase in *Bacteroidetes* (*Bacteroidaceae*) (Figs. 5.6, A.1, 5.7, A.2). Mucosal community of non-infected animals receiving probiotics (P) differed greatly from animal to animal in terms of major phyla, either dominated by *Proteobacteria* or *Firmicutes* (Figs. 5.6, A.1, 5.7, A.2). Microbiome of mice receiving empty capsules was mostly comprised of *Firmicutes* (~ 60-80% of *Lachnospiraceae* and ~ 10-20% *Ruminococcaceae*) (Figs. 5.6, A.1, 5.7, A.2; E). Infection of the group of animals that were on the same diet with *C. rodentium* led to the increase of OTUs that aligned with *Proteobacteria* up to ~ 98% in half of the animals, while in the other half ~ 30-45 % of OTUs were *Firmicutes* (*Lachnospiraceae* and *Ruminococcaceae*) (Figs. 5.6, A.1, 5.7, A.2; EC). One mouse from the group also had ~ 37 % of sequences classified as *Bacteroidetes* (Figs. A.1, A.2; EC3). Probiotic administration and infection with *C. rodentium* caused shifts in phyla composition in general towards *Proteobacteria*, however one animal had > 70% of OTUs identified as *Firmicutes* (mostly *Lachnospiraceae*) (Figs. 5.6, A.1, 5.7, A.2; PC). In PE group (probiotic capsules, no infection) mucosal community was again greatly different among animals with *Firmicutes* dominating in two mice (80-90 %), *Proteobacteria* (~ 60 %) – in one animal, and *Firmicutes*, *Verrucomicrobia* and *Bacteroidetes* comprising a community of the fourth mice (~ 20, 30 and 35 %, respectively, of the total OTUs) (Figs. 5.6, A.1, 5.7, A.2; PE). Lastly, infection of animals, fed with probiotic capsules caused a major shift towards *Proteobacteria* (> 87 % of OTUs in the communities) (Figs. 5.6, A.1, 5.7, A.2; PEC).

5.4.5.2 Analysis of the colonic mucosal community

Distances between pairs of samples were calculated, using unweighted UniFrac. Non-metric multidimensional scaling graph indicating the relationships among the individual mice is shown on Figure 5.8.

No distinct clustering of infected vs. non-infected mouse samples was found (Fig. 5.8). Samples from one of the mice from the control group (Fig. 5.8) clustered further from other controls. This was due to much higher abundance of OTUs aligning within the *Proteobacteria* (approximately 30 % of all sequences), while the other three samples were dominated by OTUs aligning within the *Firmicutes*. Also, two out of four infected controls (C2 and C4) consisted almost exclusively of OTUs homologous with *Proteobacteria* (> 95 %), while the other two had more than 90 % of sequences assigned to *Firmicutes*. All three samples from the infected group that received probiotic microcapsules (PEC) were grouped together, with all three having similar profiles with more than 80 % of their reads being identified as *Proteobacteria*.

Histopathological data from this study revealed positive correlations between the percentage of *Proteobacteria* in mucosal bacterial community and disease development (*Proteobacterial* percentage vs. average histopathological scores in both colon and cecum resulted in Spearman's correlation coefficients (R^2) > 0.400 at p -values < 0.050; Fig. 5.9, A, B). At the same time, *Firmicutes* numbers negatively-correlated with development of colitis symptoms in both parts of the intestine, though the effect was considered significant only for the colon score (Spearman's R^2 = - 0.639 at p < 0.001; Fig. 5.9, C).

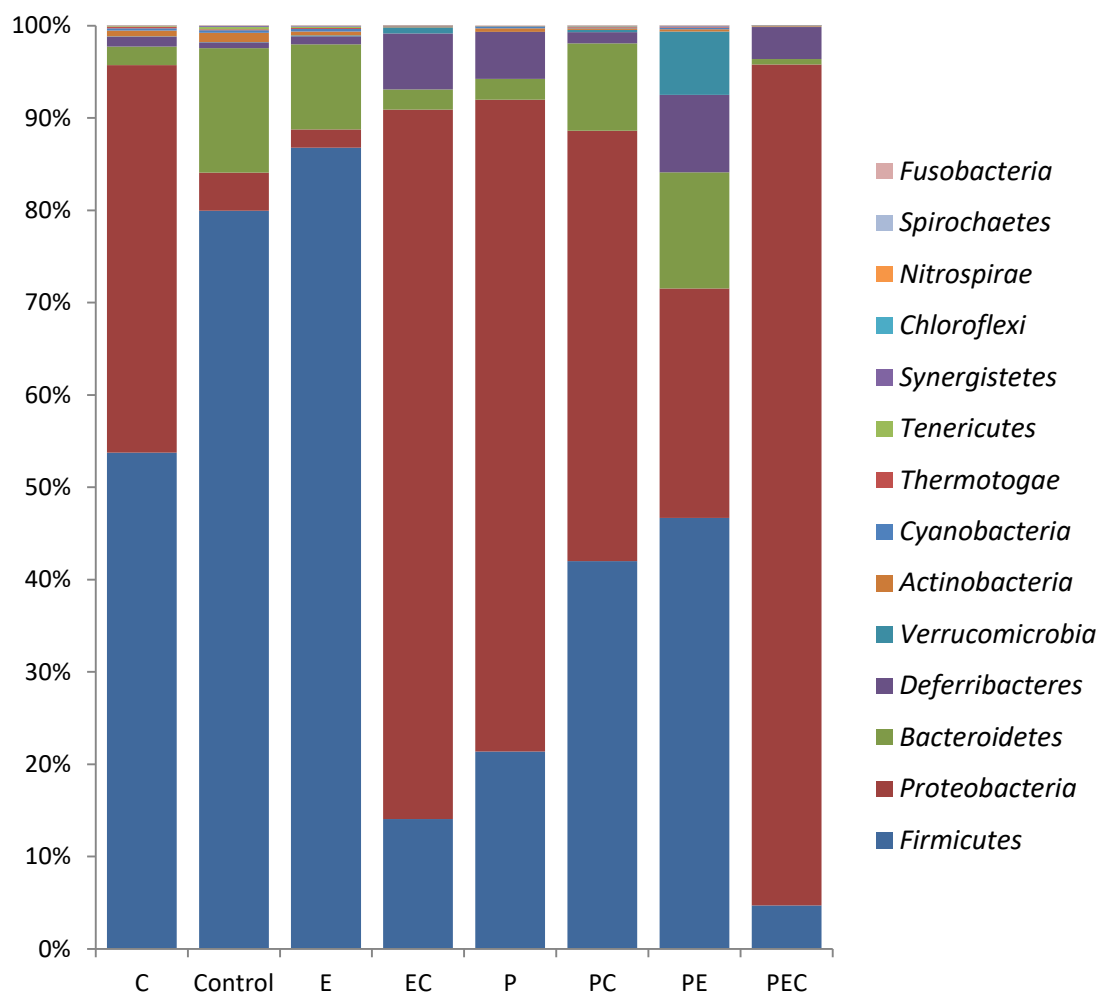


Figure 5.6. Composition of mucosa-associated community (10 dominant phyla) of distal colon of mice challenged with *C. rodentium* and/or treated with encapsulated or non-encapsulated probiotics, as sequenced on Illumina MiSeq and calculated using QIIME. The y-axis shows relative abundance. Treatment groups (n = 3-4) are shown on x-axis. Group abbreviations key: P – probiotics, C – *C. rodentium* infection, E – encapsulation.

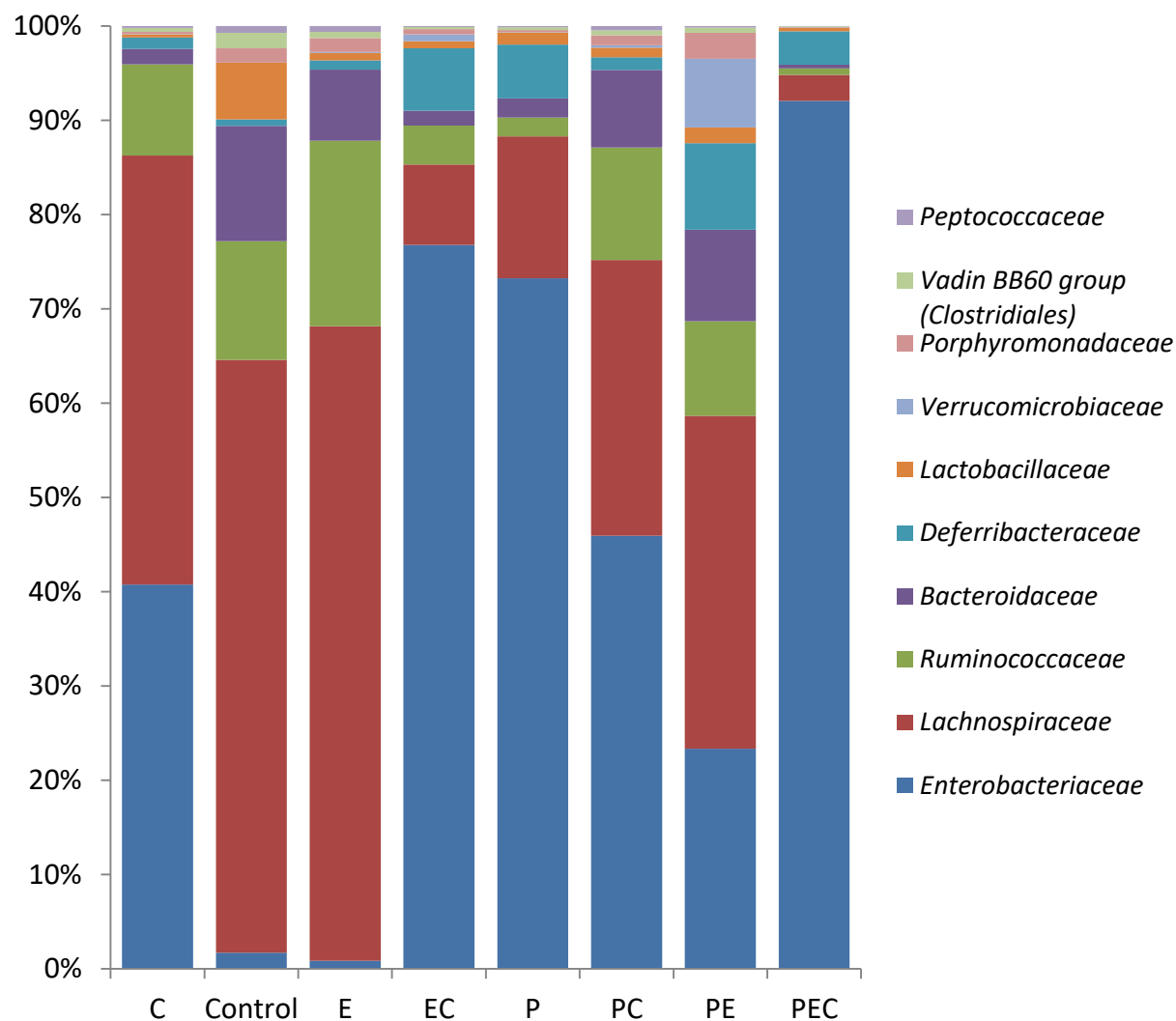


Figure 5.7. Composition of mucosa-associated community (10 dominant families) of distal colon of mice challenged with *C. rodentium* and/or treated with encapsulated or non-encapsulated probiotics, as sequenced on Illumina MiSeq and calculated using QIIME. The y-axis shows relative abundance. Treatment groups (n = 3-4) are shown on x-axis. Group abbreviations key: P – probiotics, C – *C. rodentium* infection, E – encapsulation.

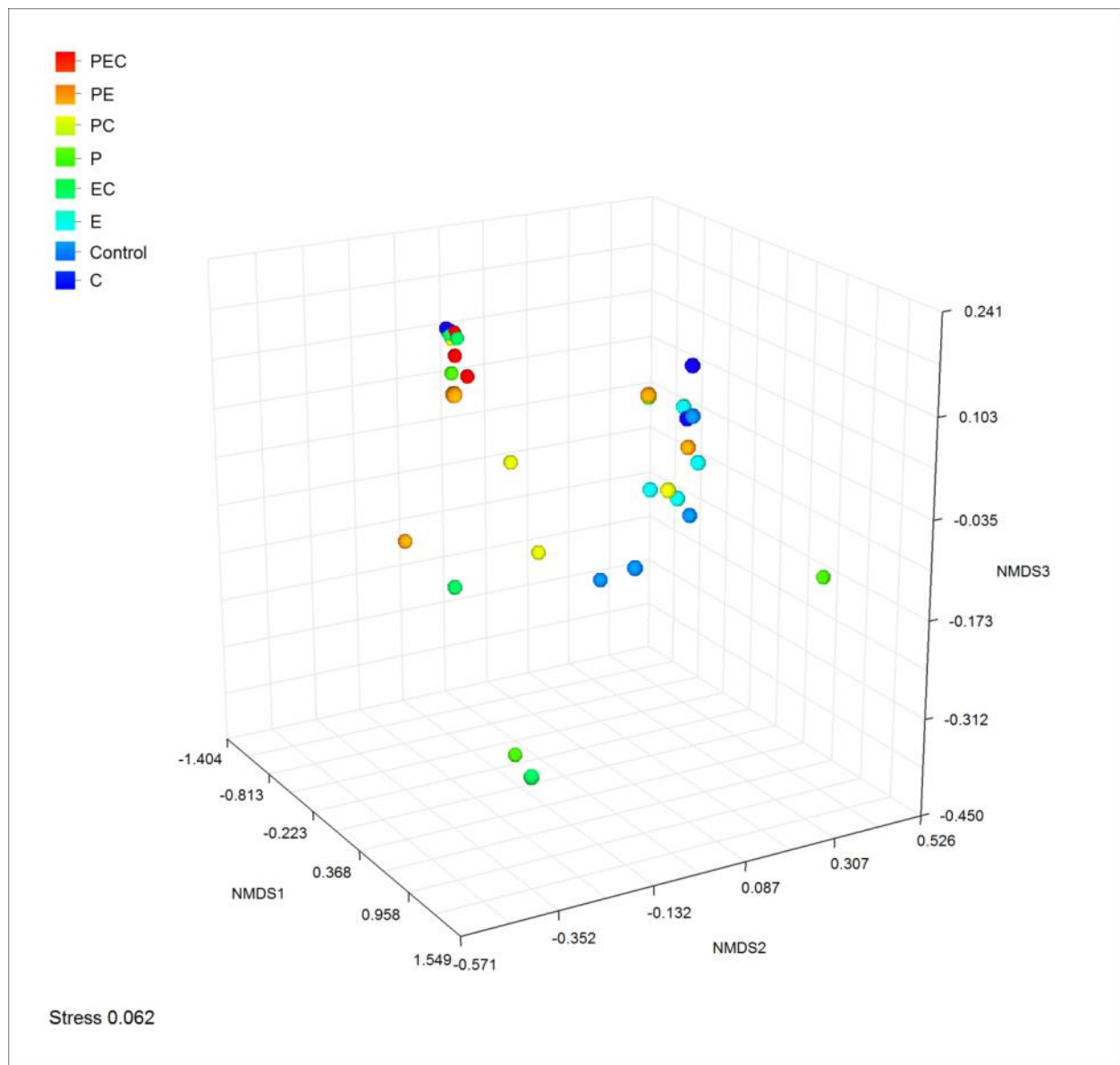


Figure 5.8. Non-metric multidimensional scaling plot, representing sample clustering and based on unweighted UniFrac dissimilarity matrix. Each sample represents a composition of mucosa-associated community of distal colon of mice challenged with *C. rodentium* and/or treated with encapsulated or non-encapsulated probiotic. Group abbreviations key: P – probiotics, C – *C. rodentium* infection, E – encapsulation.

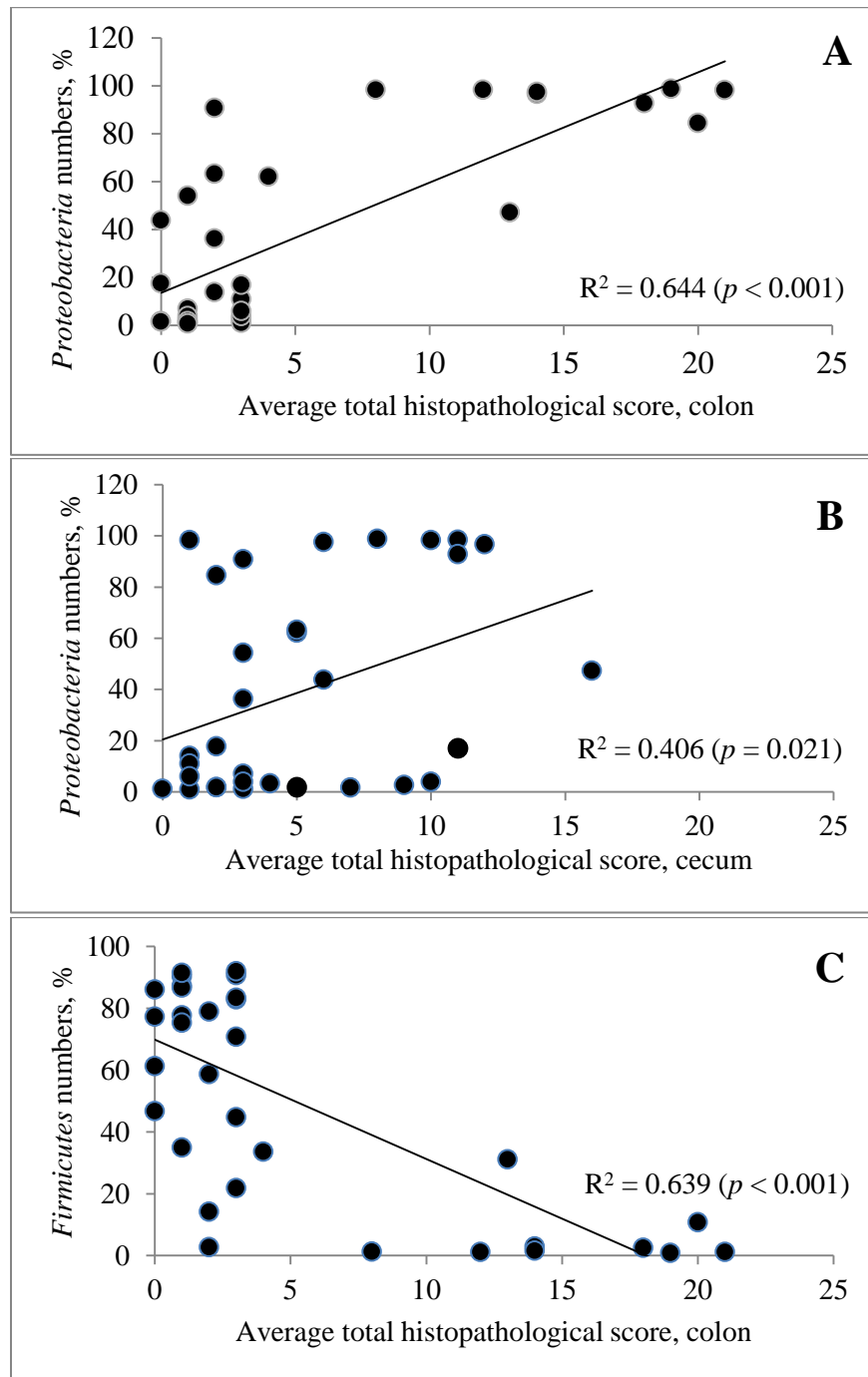


Figure 5.9. Spearman correlations between average total histopathological scores in colon (A, C) or cecum (B) and the numbers of *Proteobacteria* (A, B) and *Firmicutes* (C) (percentage of the total community) in the mucosal microbiome of distal colon of mice (n = 32).

5.5 Discussion

Infectious colitis, caused by *C. rodentium* in immunocompetent mice has been described as a good model of human EPEC/EHEC infections (Higgins *et al.*, 1999; Luperchio *et al.*, 2000; Luperchio and Schauer, 2011; Bhinder *et al.*, 2013; Chandrakesan *et al.*, 2013; Crepin *et al.*, 2016). Several studies provided well-documented evidence that *L. rhamnosus* R0011 and *L. helveticus* R0052, administered as a mixture to mice, infected with this pathogen, were able to decrease the severity of colitis, which was evident from symptomatic and histopathological data, and alteration in pro-inflammatory cytokines expression (Johnson-Henry *et al.*, 2005; Gareau *et al.*, 2010; Rodrigues *et al.*, 2012). Hence this animal model of human disease was chosen to test whether *L. rhamnosus* R0011 and *L. helveticus* R0052 would have the same beneficial effect on infected animals, when immobilized in pea protein-alginate microcapsules.

In my study, no abnormal behavior or other overt symptoms were observed in any of the animals over the course of the experiment following the *C. rodentium* challenge. Some of the observations (differences in weight, spleen size and weight, and liver weight) of disease progression in infected mice are supported by the findings of others (Johnson-Henry *et al.*, 2005; Gareau *et al.*, 2010; Costa *et al.*, 2011; Crepin *et al.*, 2016). Encapsulation had no effect on mediating any of the aforementioned symptoms. However, it should be noted that both PEC (probiotic microcapsules + infection) and EC (blank microcapsules + infection) treated mice had enlarged lymph nodes and spleens compared to those treated with the PC (freeze-dried probiotic cells + infection), which suggests that encapsulation may have exacerbated the progression of infection in this model system.

Histopathological analysis was consistent with previous studies on *C. rodentium* (Higgins *et al.*, 1999; Johnson-Henry *et al.*, 2005). While the pathogen exerted an adverse effect on various host markers, it was observed that the treatment with probiotics decreased the severity of infection. This is consistent with previous research using this disease model (Johnson-Henry *et al.*, 2005; Gareau *et al.*, 2010; Rodrigues *et al.*, 2012). However, in challenged mice, blank microcapsules also enhanced the development of the disease, particularly in the cecum. *C. rodentium* – induced colitis causes goblet cell depletion, followed by thickening of the gut mucosal layer, especially during pathogen-clearing phase (days 14 - 20 p.i.) (Gustafsson *et al.*, 2013). In response to stimuli (in this case, pathogenic infection), intestinal goblet cells may accelerate their discharge of mucin, resulting in deep cavitation of the apical membrane surface of mucoid cells and, in some cases, cause a total depletion of mucus granules making cells impossible to stain with regular histological methods

(Forstner, 1995). Barcelo *et al.* (2000) used alginate to stimulate mucin secretion and reported that exposure of rat colonic tissue to 25 mg/l sodium alginate for 30 minutes led to cavitation of large numbers of goblet cells. This outcome supports our observation of increased goblet cell depletion in the EC (blank microcapsules + infection) group of mice.

Infection with *C. rodentium* initially causes a predominantly-T_h17 immune response that later changes to a T_h1 response (Higgins *et al.*, 1999; Costa *et al.*, 2011). Probiotics *L. rhamnosus* R0011 and *L. helveticus* R0052 have been shown to suppress the expression of pro-inflammatory cytokines in this infection model (Johnson-Henry *et al.*, 2005; Rodrigues *et al.*, 2012). However, in the current study probiotic administration did not significantly impact cytokine profiles of infected animals.

One of the major functions of *Il17* is to enhance innate barrier defenses at mucosal surfaces (antimicrobial peptide production, neutrophil recruitment, etc.) (Rubino *et al.*, 2017). *Il17* receptor A-deficient (*IL17RA*^{-/-}) mice were found more susceptible to *C. rodentium* infection (Ishigame *et al.*, 2009). Here, *Il17* was expressed at high levels in the distal colon tissues of infected mice, consistent with previous work on *C. rodentium* (Johnson-Henry *et al.*, 2005; Rodrigues *et al.*, 2012). No decrease in *Il17* copy numbers was observed in infected mice fed with probiotics only as was described in the aforementioned studies. However, *Il17* was up-regulated (in comparison to the control group) in the PEC group, which suggests that the complex of encapsulation materials and probiotics had an impact on the immune system, contributing to the *C. rodentium* induced inflammation.

The pro-inflammatory cytokine – *Infγ* – promotes the activation of antigen-specific CD4⁺ T cells and macrophage phagocytosis (Shiomi *et al.*, 2010). The observed increase in expression of *Infγ* in infected animals was expected, based on previous studies of *C. rodentium* infection (Higgins *et al.*, 1999; Costa *et al.*, 2011). The concentration of *Infγ* might have not been as high as would otherwise occur due to the suppression of inflammatory response by probiotics in the PC group, and low concentrations of it in the tissue of infected control mice. High concentrations of *Infγ* in EC and PEC groups might be attributed to the effect of alginate or to the manner in which the encapsulation materials are presented to the immune system: ground calcium-crosslinked alginate mixed with protein might have had an effect similar to that of adjuvants of polysaccharide origin, though the mechanism of such effect has yet to be investigated in detail (Petrovsky and Cooper, 2011). Dobakhti *et al.* (2009) previously showed the ability of sodium alginate to induce a T_h1 immune

response in mice when used as an adjuvant for Bacillus Calmette-Guerin vaccine against tuberculosis. Also, a significant increase in the production of IFN- γ , but a decrease in the production of *Il4*, in a sodium alginate-adjuvant enhanced vaccine was reported by AbdelAllah *et al.* (2016). An indicator of a T_H2 response – *Il4* – was up-regulated in the EC group in comparison to control group. Alginate has previously been shown not to induce the expression of *Il4* (AbdelAllah *et al.*, 2016); however, this parameter may have been influenced by the presence of calcium ions, used for microcapsule crosslinking, impacting cytokine mRNA abundance in the EC group. It is a known fact that Ca²⁺ is required for the production of *Il4* by T cells and for the secretion of multiple cytokines by dendritic cells (Brown *et al.*, 1995; Li *et al.*, 2002; Parekh, 2010). Transforming growth factor-beta is a multifunctional cytokine that plays a central role in wound healing and tissue repair (Branton and Kopp, 1999; Penn *et al.*, 2012). It is also critical for T_H17 development (Mangan *et al.*, 2006). In our study, there was no significant difference in expression levels of *Tgfb1* between different treatment groups. A slightly higher (two-fold) increase in relative concentration of mRNA of this cytokine in infected animals compared to non-infected animals could be indicative of fibrosis and repair processes in the distal colon.

In general, infection of mice with *C. rodentium* caused an increase in the expression of both pro-inflammatory cytokines, as well as *Il4*; however, the effect was not uniform. The highest copy numbers of *Infy* and *Il17* mRNA were found in the distal colon of infected mice that received probiotic microcapsules (PEC), suggesting that encapsulation materials contributed to the development of inflammation. Previous work suggested that alginates may induce the immune response in rodents, especially the expression of tumor necrosis factor alpha and interleukins 1 and 6 (Otterlei *et al.*, 1991, 1993; Son *et al.*, 2001). This immuno-stimulatory effect of alginate has been linked with its interaction with the CD14 receptor on macrophages, which is similar to the activation of these cells by lipopolysaccharides (Espevik *et al.*, 1993). This effect is especially prominent in case of alginates with high mannuronic acid content (Otterlei *et al.*, 1993). Alginate, used in the current research, consisted of approximately 61 % mannuronic (along with 39 % guluronic acid for a mannuronic/guluronic acid ratio of 1.56), which could have been high enough to induce the expression of pro-inflammatory cytokines in some mice groups receiving capsules.

A very high degree of variation was observed in the structure of mucosal communities in these mice independent of treatment. However, some trends were noted. For example, similar levels of community richness and diversity were found in control mice and those fed with blank capsules.

These two metrics were affected by challenge in most of the other groups, where the microbiome profiles became more simplified (less diverse and rich). The exception was the infection control (C) group, where high within-group variability effected diversity and richness metrics. There was an obvious correlation between the numbers of *Proteobacteria* and total histopathological scores. This data is supported by observations of others, where higher *Proteobacteria* counts in animals infected with *C. rodentium* were reported (Lupp *et al.*, 2007; Berry *et al.*, 2015; Shin *et al.*, 2016). Since the PEC group was dominated by *Proteobacteria*, it may also be concluded that the impact of infection on bacterial community structure was much stronger than that of either probiotics or encapsulation materials. The composition of the microcapsules that were used for delivery of probiotics into the animal gut was pea protein and alginate, either of which could influence the mouse microbiome composition (the capsule materials comprised ~ 18 % (0.5 g) of a mouse daily dietary intake). Such an effect, though present in most of the mice in corresponding groups (E, PE, EC, PEC), was not uniform. However, alterations to mouse gut mucosal gut communities may not be as obvious as those changes which have been shown to occur in feces. Moreover, there is little information on the mucosal gut communities in mice or the extent that these may vary in terms of community composition or structure under identical experimental conditions. It is possible that within those same groups of mice, alginate could also have influenced the abundance of different bacterial groups. Alginate is considered to have low fermentability (Brownlee *et al.*, 2005). Intake of this polysaccharide usually results in development of a more simplified fecal microbiota that includes a few highly abundant phylotypes within the *Firmicutes* and an increased proportion of *Bacteroidetes* (An *et al.*, 2012; Umu *et al.*, 2015). This supports our observations of *Firmicutes* dominating the mucosal communities of E group mice. In humans, the consumption of alginate has been shown to result in a significant increase in the number of *Bifidobacteria* and a decrease in the number of *Enterobacteriaceae* (Terada *et al.*, 1995).

5.6 Conclusions

Infection of mice with *C. rodentium* led to marked progression of infectious colitis, which was evident from symptomatic and histopathological data and changes in cytokine expression. A combination of infection and probiotic microcapsules administration (PEC group) led to significantly elevated levels of both *Infy* and *Il17*, suggesting a major impact on the immune system, similar to that of an adjuvant, that triggered inflammation. EC treatment (blank microcapsules + infection) also

worsened symptoms in the cecum compared to some of the other treatments. This correlated with the upregulated expression of *Infγ* and *Il4* in the same (EC) mouse group. Administration of the probiotics *L. rhamnosus* R0011 and *L. helveticus* R0052 led to changes in most of the disease markers in the cecum in infected animals. There was no such effect observed in the colon.

Testing of the PPI-alginate two-component encapsulation matrix in a murine model of infectious colitis was challenging due to the large within-group diversity that accompanied almost every symptom or marker assessed, along with the inability to separate the effects of each of the components of the matrix. Additionally, we were not able to completely assess the interference of PPI-alginate with the ability of probiotics to lessen the severity of a disease due to the lack of such a beneficial effect of the lactobacilli strains in some infected animals. However, my results did show that PPI-alginate microcapsules used for delivery of probiotic lactobacilli worsened several symptoms of a disease and led to the induction of an inflammatory immune response in infected mice, though this effect was not uniform. Consequently, some questions remain with respect to the utilization of PPI-alginate matrix for probiotic delivery, including possible combined pathogen-ingredient inflammation responses, species-dependent responses, and the mode of action of each component under the conditions of *C. rodentium*-induced colitis in mice.

5.7 Acknowledgements

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5.8 Preface to next chapter

During the analysis of the results of the previous study it was decided to conduct a parallel experiment to investigate the ability of PPI-alginate microcapsules to protect probiotics during long

term storage. In an attempt to improve protective properties of the formulation, an extra layer of chitosan was added to the microcapsule, and the ability of PPI-alginate-chitosan to protect bacteria *in vitro* was tested.

6 SURVIVAL OF PROBIOTICS IN PEA-PROTEIN-ALGINATE MICROCAPSULES WITH OR WITHOUT CHITOSAN COATING DURING STORAGE AND IN SIMULATED GASTROINTESTINAL ENVIRONMENT³

6.1 Abstract

Pea protein–alginate microcapsules containing bacteria with or without a chitosan coating were produced by extrusion and tested for their ability to increase survivability of the bacteria during storage and an *in vitro* gastrointestinal environment. Both microcapsule formulations provided significant protection for cells incubated in synthetic stomach juice at 37°C for 2 h, followed by 3 h in simulated intestinal fluid, relative to non-encapsulated bacteria. However, evaluation of cell viability during 9 weeks of storage at room temperature revealed that chitosan coating significantly improved microcapsule ability to protect bacteria compared to non-coated microcapsules. Refrigerated storage had no negative impact on capsule protective ability in both types of capsules. Notably, chitosan-containing microcapsules showed much higher viable counts during challenge tests even after the storage. Moreover, the addition of chitosan to the microcapsule formulation was not observed to increase the microcapsule size.

6.2 Introduction

In 2001, FAO/WHO experts defined probiotics as living microorganisms that confer a health benefit when administered in adequate amounts (FAO/WHO, 2001). Although there have been numerous studies describing the potential of probiotics in the prevention and treatment of multiple gastrointestinal (GIT) disorders, the efficacy of these microbial strains remains arguable. Probiotic-based strategies for therapeutic and prophylactic use against GIT diseases have been recently reviewed by our group (Varankovich *et al.*, 2015b). Apart from the challenge of finding the right species that would ‘confer a health benefit’ to its host, there are also technical issues, such as

³Varankovich, N., Martinez, M. F., Nickerson, M. T., and Korber, D. R. (2016). Survival of probiotics in pea-protein-alginate microcapsules with or without chitosan coating during storage and in simulated gastrointestinal environment. *Food Sci Biotechnol* 26, 189-194. doi:10.1007/s10068-017-0025-2

delivery of the probiotic to a certain part of GIT where it could perform its function. One way to circumvent this issue is through the immobilization of bacteria in a protective polymer matrix. Microencapsulation of probiotic bacteria has become a widely and successfully used in the protection of acid-sensitive bacteria during storage and/or passage through a simulated GIT environment (Chandramouli *et al.*, 2004; Jiménez-Pranteda *et al.*, 2012; Khan *et al.*, 2013). With respect to materials used for encapsulation it is preferable to choose plant proteins (soy, pea) and natural biopolymers, such as alginate or carrageenan, that are generally recognized as safe (GRAS) and are able to provide a good protection for bacterial cells (Holzapfel *et al.*, 2002; Annan *et al.*, 2008; Varankovich *et al.*, 2015a). However, since not all of such materials provide sufficient protection by themselves, there has been a growing interest in using various mixtures, coatings and layers of different biopolymers to increase viability of encapsulated bacteria.

Sodium alginate, pea protein isolate and chitosan, used for microencapsulation in this study, have GRAS status. Sodium alginate is a polymer of algal origin, composed of (1→4)-linked β-D-mannuronic (M) and α-L-guluronic acid (G) residues, and undergoes gelation in the presence of divalent calcium ions, making it ideal for the extrusion-based encapsulation process (Voo *et al.*, 2011). The use of pea protein isolate (PPI) in the food industry is gaining popularity as an alternative to both animal-derived proteins and to soy, a known allergen. Pea proteins are comprised of mainly of globulin-type proteins of molar mass of 320-400 kDa (legumin) and 150 kDa (vicilin), and albumin (Stone *et al.*, 2015). Chitosan (β-(1→4)-poly-D-glucosamine) is a biodegradable, biocompatible, nontoxic and non-allergenic linear cationic polysaccharide derived from chitin via deacetylation (Türkoğlu and Taşcıoğlu, 2014). It is widely used in medicine, agriculture, functional foods, biotechnology and cosmetics (Wang *et al.*, 2012). For encapsulation, typically a gel-like capsule matrix is initially formed, followed by the addition of the coating. The binding of the coating material in the proposed capsule formulations will be due to polyelectrolyte complexation involving the positively-charged chitosan reacting with negatively-charged alginate (Zheng *et al.*, 2016).

My earlier research (chapter 3) has shown that PPI-alginate microcapsules have the potential to protect acid-sensitive bacteria *in vivo* (Varankovich *et al.*, 2015a). In attempt to improve microcapsule formulation I added chitosan to the mixture, since this polymer has been shown by others to improve protective properties of microcapsules made of alginate (Chávarri *et al.*, 2010). The bacteria used in the study were *Lactobacillus rhamnosus* R0011 and *L. helveticus* R0052 (mixed

in 95:5 ratio; Lacidofil®; Lallemand Health Solutions, Montreal, QC, Canada), whose probiotic potential in suppressing *Citrobacter rodentium* infection in mice has previously been reported (Gareau *et al.*, 2010; Rodrigues *et al.*, 2012). The goal of this study was to examine the ability of chitosan coating to improve protective properties of PPI-alginate microcapsules and also to determine how different storage conditions affect the viability of bacteria entrapped in both types of capsules.

6.3 Materials and methods

6.3.1 Materials

Pea protein isolate was donated by Nutri-Pea Ltd. (Portage La Prairie, MB, Canada). Alginic acid sodium salt, calcium chloride dehydrate, Tween 80 and chitosan (low molecular weight) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Probiotic strains *L. rhamnosus* R0011 and *L. helveticus* R0052 were received from Lallemand Health Solutions (Montreal, QC, Canada).

6.3.2 Bacteria and culture conditions

Probiotic strains were stored at -80°C in a 1:2 (v/v) suspension of glycerol and de Man, Rogosa and Sharpe broth (MRS; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Prior to bacterial encapsulation, frozen stocks were plated on MRS medium containing 1.5 % (w/v) agar and then incubated for 24 h at 37°C under anaerobic conditions (80 % N_2 , 10 % CO_2 and 10 % H_2) in an anaerobic chamber (Forma Scientific Inc., Marietta, GA, USA). Pure colonies of *L. rhamnosus* R0011 and *L. helveticus* R0052 were then transferred from plates to 50 mL tubes containing 45 mL of MRS broth for incubation under the same conditions for 24 h. Bacterial cells were then recovered by centrifugation ($2000\text{ g} \times 12\text{ min}$) (Sorvall SS-1; Sorvall Inc., Newtown, Connecticut, USA), washed twice with 1.0 % alkaline peptone water (APW; 10 % (w/v) peptone, 10 % (w/v) sodium chloride, pH 8.5) and used for the preparation of microcapsules.

6.3.3 Preparation of bacteria-containing microcapsules

Encapsulation and extrusion techniques used in this study were based on methodology described by Krasakoopt *et al.* (2003), Chávarri *et al.* (2010) and Klemmer *et al.* (2011) with modifications. The composition of the capsule material was as follows: 2.0 % PPI + 0.5 % (w/w) sodium alginate. Concentration of PPI was adjusted to the protein content of the isolate (85 %). PPI

was dissolved at 85°C and pH 9.0 for 2 h under constant mechanical stirring. The pH was then adjusted to 7.0 and sodium alginate powder was added to the mixture. After complete dissolution of the polysaccharide (1–1.5 h at 85°C), the solution was cooled to room temperature (21–23°C), corrected for evaporative water loss with the addition of sterile water and mixed with a 2.0 % (v/v) bacterial slurry preparation (*L. rhamnosus* R0011 and *L. helveticus* R0052 at a 95:5 ratio). The final mixture of protein, polysaccharide and bacteria was then extruded using a syringe and 18 G (G, gauge) needle into 200 mL of ddH₂O containing Ca²⁺ ions (cross-linking agent) and Tween 80 (emulsifier). The concentrations of Ca²⁺ (CaCl₂) and Tween 80 were 1.0 % (w/v) as previously described (Varankovich *et al.*, 2015a). A portion of microcapsules were extruded into a cross-linking solution of the same composition, but with the addition of chitosan (0.4 %, w/v). After extrusion, microcapsules were left in the cross-linking solution on a shaker for 15 min, removed by filtration and left to air-dry for 15 min, then afterwards freeze-dried (Labconco FreeZone, Kansas City, MO, USA) for 15 h in the absence of cryoprotectants.

6.3.4 Viability of bacteria in microcapsules during storage

Both types of microcapsules – with and without chitosan coating were stored for 9 weeks in bags (200 x 300 mm) of plastic barrier film (75 micron nylon/polyethylene, oxygen transmission rate 63 cc/sq. m/24 h at 23°C) in triplicate under four different conditions: 4°C, 20°C, 4°C + vacuum (KOMET Vakuun-Verpacken, KOMET Maschinenfabrik GmbH, Plochingen, Germany), and 20°C + vacuum. Aliquots of microcapsules (0.1 g) were removed from bags every week, homogenized (Omni-mixer, Sorvall Inc., Newtown, USA) at 2000 rpm for 2 min, serially diluted, plated on MRS agar in triplicate, incubated at 37°C for 48 h and enumerated. Results were presented as log CFU/g of microcapsules.

6.3.5 Survival of probiotics under simulated stomach conditions

To determine the survival of encapsulated bacteria before and after the storage period (on weeks 0 and 9) 0.1 g aliquots of microcapsules were added to 0.9 ml of synthetic stomach juice (SSJ; 8.3 g/L proteose peptone, 3.5 g/L glucose, 2.05 g/L NaCl, 0.6 g/L KH₂PO₄, 0.11 g/L CaCl₂, 0.37 g/L KCl, 0.05 g/L bile salts, 0.1 g/L lysozyme, 13.3 mg/L pepsin; pH 1.8) (Krasaekoopt *et al.*, 2003; Khan *et al.*, 2013) and incubated for 2 h at 37°C, then transferred to 0.9 ml of simulated intestinal fluid (SIF; 1.25 % (w/v) NaHCO₃, 0.60 % (w/v) bile salts, 0.09 % (w/v) pancreatin; pH 6.5) and

incubated for another 3 h at 37°C. By the end of incubation all microcapsules were completely dissolved in SIF, and numbers of live bacteria cells were determined by serial dilutions in 1.0 % APW and plating on MRS agar. The same method, but without exposure to SSJ, was used to quantify the initial cell counts within microcapsules.

To assess the viability of planktonic cells in SSJ, 0.01 g aliquots of lyophilized bacteria were added to centrifuge tubes (2 mL) containing 0.99 mL of SSJ and incubated at 37°C. Starting at time 0, then every 5 min over the first 30 min, and subsequently every 30 min (2 h in total), tubes were removed from the incubator, serially-diluted with 1.0 % APW and plated on MRS agar. Plates were incubated at 37°C in an anaerobic chamber for 48 h and enumerated. All experiments were conducted in triplicate.

6.3.6 Morphology and size of capsules

Lyophilized microcapsules were cut in half using a scalpel, stained and immediately immobilized on the surface of a cover glass using molten 0.8 % (w/v) agar tempered to 40–45°C. After the agar solidified, the cover glass was inverted and transferred to a glass chamber made from glass slides. The chamber was then filled with tempered liquid agar. Staining of the microcapsules was performed using SYTO 9 and propidium iodide (5 mM solutions in DMSO; Life Technologies™ Corp., Carlsbad, CA, USA) according to the guidelines provided by Life Technologies™ (SYTO 9 and propidium iodide manuals, 2011). Dyes (1 µL each) were added to 2 mL water, vortexed, directly applied to the capsule surface and then incubated for 15 min in the dark. Bright field and fluorescent images of stained microcapsules were obtained using a Nikon C2 confocal laser scanning microscope (CLSM; Nikon, Mississauga, ON, Canada) using 488 nm and 543 nm wavelength excitation in conjunction with an oil immersion, 60X Plan Apo VC (1.4 N.A., Nikon) objective lens. Three capsule locations were randomly chosen for obtaining optical thin sections representative of the capsule surface and stained bacteria. Each image was taken with a xy-resolution of 512 by 512 pixels and a pinhole radius of 20 µm.

Microcapsule size was measured using an electronic digital caliper (model 62379-531, Control Company, Friendswood, TX, USA). Fifteen microcapsules for each capsule type were measured and averaged.

6.3.7 Statistics

Mean values, standard deviation, t-values and p-values within each group, as well as between groups, were determined using Welch *t*-test and Two-Way ANOVA (One-Way ANOVA in case of capsule size analysis). The level of statistical significance was set at $p < 0.05$. All statistical analyses were performed using SPSS software (SPSS Inc., Ver. 17.0, 2008, Chicago, IL, USA).

6.4 Results and discussion

6.4.1 Protective properties of microcapsules during storage

The viability of encapsulated bacteria was monitored over a period of 9 weeks to determine the effect of temperature and vacuum packaging on their survival for both PPI-alginate and PPI-alginate-chitosan microcapsules (Fig. 6.1). Results showed that cell viability in both types of capsules improved ($p = 0.001$) when stored at 4°C compared to 22°C. When stored at room temperature, chitosan-coated microcapsules provided better protection for cells compared to plain PPI-alginate capsules. There was a decrease in bacterial viability of 0.7 log CFU/g (from initial 8.10 log CFU/g) in PPI-alginate-chitosan microcapsules stored at 22°C by week 9, while viable counts in PPI-alginate microcapsules, stored under the same conditions, reached 6.68 log CFU/g by week 5 (Fig. 6.1). By week five, viable cell numbers continued to decrease, but plating results started to fluctuate considerably and are not shown on the graph (Fig. 6.1A). Vacuum sealing did not have any effect on cell survival in either PPI-alginate or PPI-alginate-chitosan microcapsules stored at room temperature ($p > 0.05$; Fig. 6.1A, B). However, stored PPI-alginate-chitosan microcapsules held under vacuum at 4°C, elicited no reduction in viable bacterial numbers (Fig. 6.1B).

These findings are in accordance with other studies, where it was reported that stability of dried cultures decreased significantly when stored at room temperature (Champagne and Mondou, 1996; Foerst *et al.*, 2012). Obradović and Krunić (2015) also observed greater survival of bacteria in chitosan-coated alginate beads, compared to non-coated ones, during 21 days of refrigerated storage (4°C). Chávarri *et al.* (2010) reported that bacterial numbers in chitosan-coated alginate microcapsules decreased by 2.0 log CFU/g over 28 days of storage at 4°C. However, our results showed considerably less viability loss in pea protein alginate microcapsules, coated with chitosan – only 0.3 log CFU/g during 9 weeks of refrigerated storage (Fig. 6.1B). Improved protective properties might be caused by the addition of pea protein isolate to the formulation. According to the results, the best way to preserve viable probiotic cells of *L. rhamnosus* R0011 and *L. helveticus*

R0052 for 9 weeks was encapsulating them in PPI-alginate with chitosan coating and storing at 4°C under vacuum.

6.4.2 Survival of probiotic bacteria in microcapsules during simulated GIT conditions

Free non-encapsulated bacterial cells were incubated in synthetic stomach juice (SSJ) for 2 h as a control, where viable cells became undetectable by plating after 60 min (*L. rhamnosus* R0011) and 90 min (*L. helveticus* R0052) incubation (Fig. 6.2). To assess the survival of immobilized bacteria under simulated gastrointestinal tract conditions after 9 weeks of storage, PPI-alginate and PPI-alginate-chitosan microcapsules were incubated for 2 h in synthetic stomach juice (SSJ) and then for 3 h in simulated intestinal fluid (SIF). Cell viability within freshly prepared PPI-alginate and PPI-alginate-chitosan (control) and after incubation 2 h in SSJ and 3 h in SIF revealed that PPI-alginate-chitosan microcapsules provided better protection, with only having a decrease in viable counts by 0.7 log CFU/g compared to a 1.5 log CFU/g decline in PPI-alginate microcapsules after the 5 h test ($p < 0.05$) (Fig. 6.3).

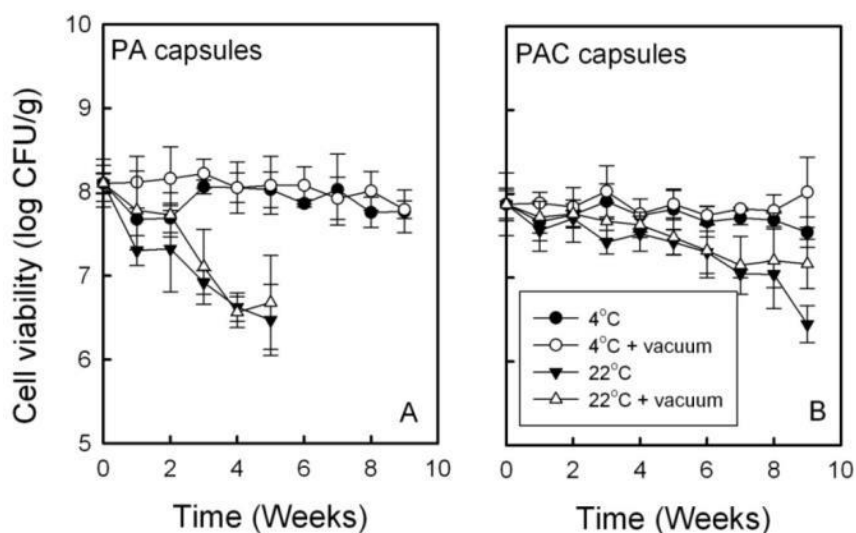


Figure 6.1. Viability of probiotic bacteria in pea-protein-alginate (A) and PPI-alginate-chitosan (B) microcapsules during storage at 4 and 22°C without and under vacuum over a 9-week duration. Data represent the mean \pm one standard deviation ($n = 3$).

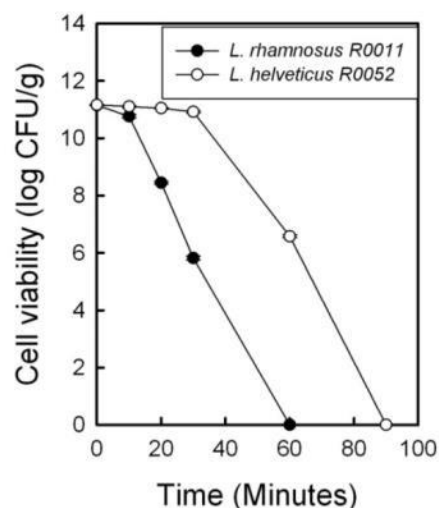


Figure 6.2. Decrease in viable cell counts of non-encapsulated probiotic bacteria (*L. rhamnosus* R0011 and *L. helveticus* R0052) during incubation 2 h in synthetic stomach juice. Data represent the mean \pm one standard deviation (n = 3).

Viability tests were repeated after 9 weeks of storage under different temperature (4 or 22°C) and atmospheric (with and without vacuum) conditions (Fig. 6.4). Microcapsules were tested for cell viability before (control) and after incubation within SSJ (2 h) followed by SIF (3 h). PPI-alginate microcapsules stored at room temperature provided the least protection for immobilized cells, with a 4.1 log CFU/g decrease in viable counts (Fig. 6.4A). Storage at 22°C under vacuum did not improve their performance (Fig. 6.4A). When stored at 4°C, cell viability was improved relative to the room temperature storage, and losses in cell viability were only 1.3 log CFU/g and 1.9 log CFU/g with or without vacuum, respectively (Fig. 6.4A). The addition of a chitosan coating to the PPI-alginate microcapsules led to improved cell viability ($p < 0.05$), where no losses cell viability was observed after treatment with SSJ + SIF in samples stored under different temperature (4 or 22°C) or atmosphere (with or without vacuum) conditions (Fig. 6.4). Zanjani *et al.* (2013) reported that chitosan coating played a significant role in the protection of *Lactobacillus casei* ATCC 39392 and *Bifidobacterium bifidum* ATCC 29521 immobilized in calcium-alginate gelatinized starch microcapsules during simulated gastric- and intestinal juices challenge test. Chitosan-coated alginate beads similarly provided good protection for *L. acidophilus* and *L. casei* cells during survival tests as reported by Krasaekoopt *et al.* (2004).

Protection of bacterial cells immobilized in PA microcapsules is mainly provided by an outer membrane that is formed upon extrusion of the microcapsules material into a crosslinking solution containing divalent cations. The improvement in the protective properties of PAC microcapsules compared to those consisting of PA only can be attributed to an additional polyelectrolyte complex membrane that forms on the surface of negatively charged alginate when positively charged chitosan diffuses into its three-dimensional network (Zheng *et al.*, 2016).

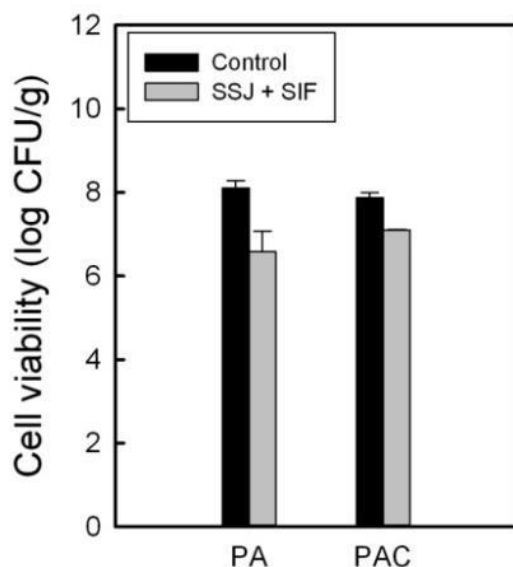


Figure 6.3. Cell viability within freshly prepared PPI-alginate (PA) or PPI-alginate-chitosan (PAC) microcapsules (control)) and after incubation for 2 h in synthetic stomach juice (SSJ) followed by 3 h in simulated intestinal fluid (SIF). Data represent the mean \pm one standard deviation ($n = 3$).

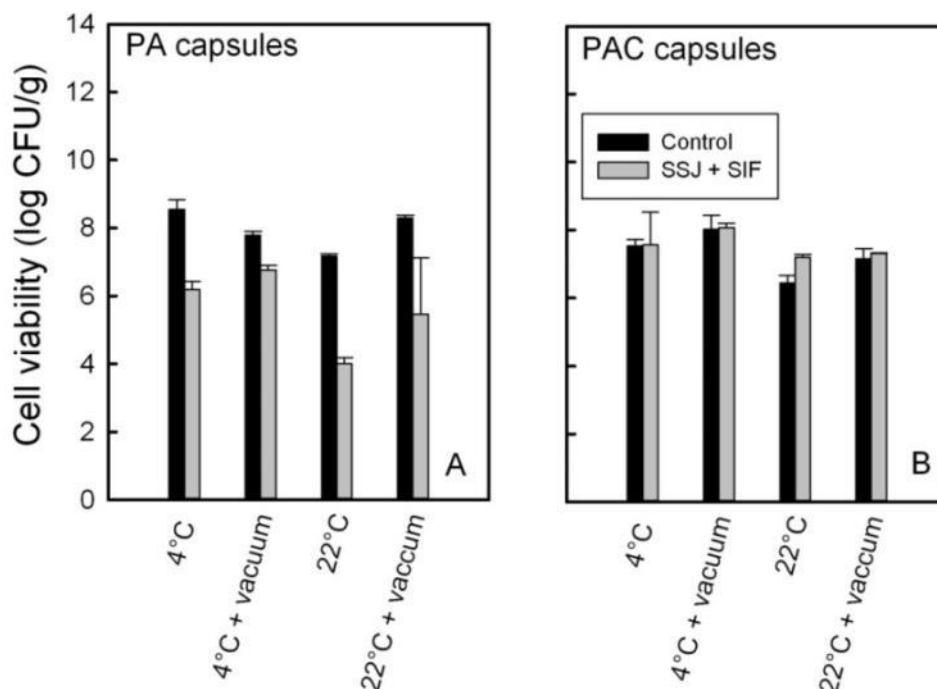


Figure 6.4. Cell viability in PPI-alginate (PA; A) and PPI-alginate-chitosan (B) microcapsules after 9 weeks of storage at 4 and 22°C without and under vacuum, before (control) and after incubation for 2 h in synthetic stomach juice (SSJ) followed by 3 h in simulated intestinal fluid (SIF). Data represent the mean \pm one standard deviation ($n = 3$).

6.4.3 Microscopy and size of microcapsules

The morphology of the surface of PPI-alginate and PPI-alginate-chitosan microcapsules were imaged using CLSM (Fig. 6.5). No discernable differences could be seen with the addition of chitosan, since the coating thickness was too thin to be visualized by this technique which has a minimum resolving power of 0.2 microns. On average, the size of PPI-alginate microcapsules was ~ 2.4 mm, where the addition of the chitosan coating had little impact on capsule diameter (Fig. 6.6). However, after incubation in SSJ for 2 h, the diameters of both types of microcapsules became reduced slightly to approximately 2.2 mm, although this was not considered significant ($p > 0.05$) (Fig. 6.6). Alginate (only) microcapsules have previously been shown to shrink at low pH (Ouweryx *et al.*, 1998; Valenzuela *et al.*, 2016). Alginate molecules have a pKa of ~ 3.5 and therefore they lose 50 % of their charge at pH values less than 5.5, leading to a dense structure and shrinkage of alginate-based microcapsules (Valenzuela *et al.*, 2016).

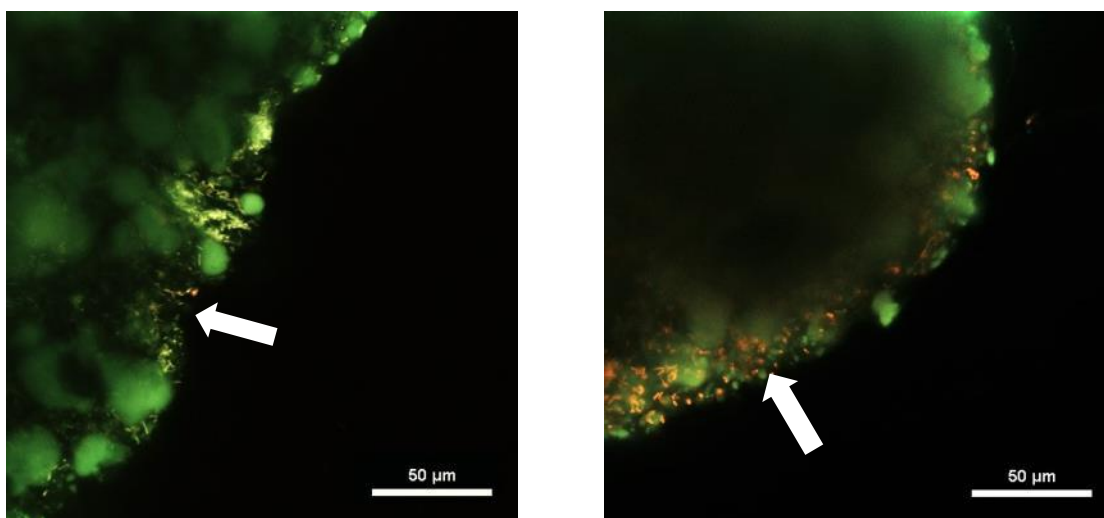


Figure 6.5. CLSM images of the surface of PPI-alginate (left) and PPI-alginate-chitosan (right) microcapsules stained with propidium iodide and SYTO 9. Arrows indicate entrapped bacteria. Scale bar – 50 μm .

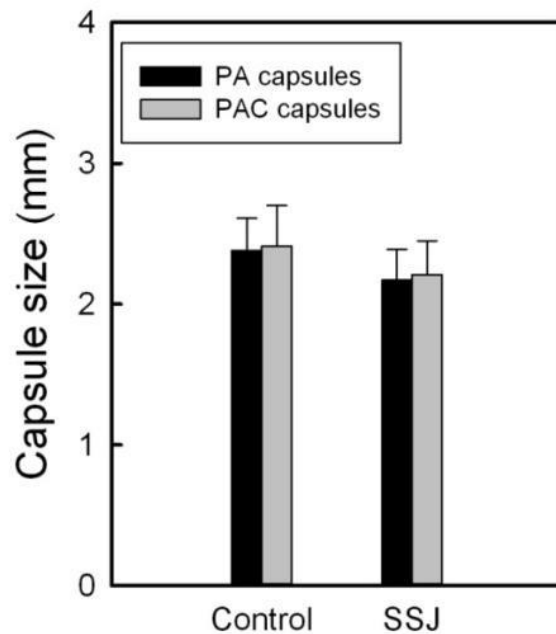


Figure 6.6. Diameter of PPI-alginate and PPI-alginate-chitosan capsules, before and after incubation for 2 h in synthetic stomach juice (SSJ). Data represent the mean \pm one standard deviation ($n = 15$ for each capsule type).

6.5 Conclusions

To conclude, PPI-alginate microcapsules formulated with and without a chitosan coating enhanced the survival of probiotic cells during refrigerated (4°C) storage for 9 weeks, with little differences observed in cell viability before and after incubation in simulated gastrointestinal condition (SIF + SSJ) challenge. In contrast, storage at 22°C worsened the performance of both types of capsules. While addition of chitosan to the microcapsule formulation significantly improved storage and viability during simulated GIT challenges, the coating was so thin it couldn't be visualized using CLSM. Vacuum packaging did not have any significant impact on the protective properties of microcapsules stored at room temperature, but improved cell survival at 4°C in PPI-alginate-chitosan microcapsules. These results suggest that PPI-alginate microcapsules coated with chitosan can be successfully used to preserve high viable counts of *L. rhamnosus* R0011 and *L. helveticus* R0052 during long-term refrigerated storage.

7 GENERAL DISCUSSION

The delivery of live probiotics in sufficiently high numbers to the intestine remains a major challenge. Probiotic products should contain substances that protect viable bacteria from detrimental conditions of the stomach so that they can reach the colon in sufficient number in order to provide a functional benefit to the host. The most efficient method for protection of bacteria from harmful influence of acid and bile is their encapsulation within biopolymer coatings, and preference has usually been given to natural hydrocolloids and gums that are generally recognized as safe for human health and show good protection of bacterial cells against the acidic environment of the gut. Examples of such polysaccharides used for encapsulation include alginate, carrageenans and gums, all of which possess acceptable properties of viscosity, water binding, emulsion stabilization, adhesion, foam stabilization and film formation (Holzapfel and Schillinger, 2002; Shah *et al.*, 2007; Bajaj *et al.*, 2007; Chaplin, 2012; Morris *et al.*, 2012). Previously, Wood *et al.* (2010) found that alginate capsules (1.0 %; w/v) were unable to provide sufficient protection to *B. adolescentis* ATCC 15703 during a 2 h incubation in simulated gastric juice, an outcome that was attributed to the presence of large pores (~ 400 µm) on the surface of the alginate capsules. However, Lee and Heo (2000) reported only a 2 log CFU/mL decrease in viable cell numbers of *Bifidobacterium longum* encapsulated in 2.0 % (w/v) alginate under similar conditions. This difference might be explained by the fact that *B. longum* has a higher degree of acid tolerance, as different species of bifidobacteria have varying sensitivities to acidic conditions (Waddington *et al.*, 2011).

In my research, microcapsules with immobilized *B. adolescentis*, and containing PPI-alginate, PPI-*iota*-carrageenan or PPI-gellan gum, demonstrated significant retention of viability in SSJ compared to planktonic cells. This is in agreement with the results of Klemmer *et al.* (2011), who reported that pea protein isolate-alginate microcapsules prepared using extrusion offered protection to *B. adolescentis* in simulated gastric juice over 2 h at 37°C, and showed prolonged release in simulated intestinal fluids. Wang *et al.* (2015) showed that *B. adolescentis* cells trapped in pea, soy, faba, and lentil protein-alginate microcapsules showed 1.9, 3.3, 5.1, and

5.5 log reductions in cell numbers, respectively, after a 2 h challenge. In our experiments pea-protein alginate microcapsules showed slightly better performance in SSJ – live cell count decreased by ~ 1 log CFU/g for fresh microcapsules. As for release of entrapped bacteria, Wang *et al.* (2015) reported the release of almost all bacterial cells encapsulated in legume protein-alginate microcapsules after 10 min in SIF, which also supports our data for PPI-alginate capsules. Interestingly, increasing of the concentration of PPI to 4.0 % led to decreased viable counts of *B. adolescentis* after 2 h in synthetic gastric juice (Wood, 2010). Even better results were achieved with *iota*-carrageenan (74 % of cells released, compared to 70 % for PPI-alginate). In contrast, PPI-gellan gum microcapsules released far fewer cells (~ 40 %) due to the greater resistance of PPI-gellan gum complex to degradation by SIF.

Alginate and *iota*-carrageenan have been previously described to have good freeze/thaw stability and potential for cell protection during lyophilization, which is an important method for probiotic preservation for long-term storage (Giulio *et al.*, 2005; Gbassi and Vandamme, 2012). In this research, the effect of freeze-drying on the protective properties of microcapsules was also tested using the same above-described methodology. All three types of lyophilized microcapsules provided sufficient protection for bacterial cells from the detrimental effect of SSJ compared to planktonic cells, with no significant difference in cell viability observed amongst different types of microcapsules (~ 2-2.5 log reduction in CFU/g). Freeze-dried microcapsules released slightly more viable bacteria than fresh microcapsules (79 %, 75 % and 53 % of entrapped cells for PPI-*iota*-carrageenan, PPI-alginate and PPI-gellan gum capsules).

Treatment with SSJ caused PPI-*iota*-carrageenan microcapsules to slightly decrease in size and change in shape. Contrary to the reports of others (Klemmer *et al.* 2010; Wang *et al.* 2015), there was no significant shrinking of PPI-alginate observed at low pHs. After transfer to SIF, both fresh and dried PPI-alginate and PPI-*iota*-carrageenan microcapsules became dissolved in the solution after 3 h; however, PPI-gellan gum microcapsules of both types did not dissolve, but did change shape and size, becoming spherical. The dissolution of PPI-alginate and PPI-*iota*-carrageenan microcapsules under simulated conditions of lower GIT is an asset since it allows release of all the immobilized bacteria. A possible reason for the dissolution of PPI-*iota*-carrageenan matrix might be disruption of electrostatic bonds existing between protein and polysaccharide. Since PPI has an isoelectric point (pI) of ~ 4.5, at pH values < pI the protein would assume a net-positive charge, while all polysaccharides used in the study would carry a negative charge at pH values >

1.88 (pK_a of $-\text{COO}^-$) in the case of gellan gum and alginate, and > 2.00 (pK_a of $-\text{SO}_3^-$) in the case of *iota*-carrageenan. According to Ganzevles (2007), when the polysaccharide $\text{pK}_a < \text{pH} < \text{pI}$ (protein), both components would be oppositely-charged, and thus they would be electrostatically-attracted to each other. Hence, under simulated intestinal conditions ($\text{pH } 6.5$), both protein and polysaccharide would have the same charge and thus would repel each other, and the structural integrity of a capsule would be maintained only by the calcium-stabilizing polysaccharide network. Additionally, *iota*-carrageenan has a greater net-negative charge (due to higher electronegativity of its sulphate groups), which led to higher repulsive forces within the microcapsule, followed by a disruption of the calcium-carrageenan network and a release of the majority of entrapped bacteria into the solution. These findings are supported by the studies of polymer and drug release characteristics from alginate capsules (Kim and Lee, 1992; Sugawara *et al.*, 1994; Chen *et al.*, 2004; Chen *et al.*, 2007). It has also been shown that at low pH the surface of alginate capsules becomes porous and insoluble (George and Abraham, 2006), while in higher pH solutions this so-called “alginic acid skin” becomes soluble, and alginate starts to swell, which, in the case of probiotic capsules, might aid in the controlled release of bacterial cells (Kimura, 1993). In general, alginate gels are considered fragile, since they reveal an open cellular structure after drying (Nussinovitch *et al.*, 2000). The stability of such gels can be preserved by using lyophilization as a dehydration method, as it results in dry gels with improved mechanical properties (Nussinovitch *et al.*, 1993). The addition of pea protein to alginate also considerably improved their stability under acidic conditions, allowing effective protection of immobilized bacteria.

Confocal microscopy (CLSM) images of all three types of fresh microcapsules (stained and bisected) revealed that the cells were evenly distributed within the capsules, immobilized on the surface of pea protein particles and within the unstained polysaccharide. After freeze-drying protein globules were slightly deformed and surrounded by layers of polysaccharide containing immobilized bacteria, forming pores separated by protein-polysaccharide walls. These pores could be the result of ice sublimation, occurring during lyophilization (Shamblin *et al.*, 2004). Such honeycomb-like ultrastructure has been previously described by Khan *et al.* (2013) and Wood (2010), based on scanning electron microscopy (SEM) and cryo-SEM of PPI-alginate capsules. However, as evident from the survival studies in this research, pores apparently do not facilitate the diffusion of SSJ into the capsules, as the viability of bacteria within both freeze-dried- and fresh microcapsules was approximately equal.

Culture-dependent methods for bacterial enumeration cannot be applied to the quantification of most of the bacterial species found in GIT due to the absence of a suitable medium, which makes culture-independent techniques, such as PCR, a required tool for this purpose (Giraffa and Carminati, 2008). PCR have already been successfully used for the quantification of bacteria (including *B. adolescentis*) in human and animal feces (Wang *et al.*, 1996; O'Sullivan, 1999). In the study by Satokari *et al.* (2001), a group of human subjects were administered *Bifidobacterium lactis* at a concentration of 3×10^{10} CFU per day for two weeks. The quantification of *B. lactis* in feces was conducted using PCR/gel electrophoresis techniques. However, no *B. lactis* were detected after the administration was ceased (Satokari *et al.*, 2001). In the current study, the results of PCR with *B. adolescentis*-specific primers, followed by gel electrophoresis, demonstrated an increased abundance of *B. adolescentis* in the GIT of rats that were fed PPI-alginate microcapsules containing bacteria. Additionally, similar with the data reported by Satokari *et al.* (2016), the elimination of fed probiotic bacteria from the animal GIT occurred within one-week post-supplementation.

The effect of encapsulation materials on the GIT as well as their possible interference with the action of encapsulated probiotics has to be investigated as well. In this research, the animal model of infectious disease (*C. rodentium*-induced colitis) was used to test the effects of PPI-alginate matrix on the action of probiotics *L. rhamnosus* R0011 and *L. helveticus* R0052. To the best of my knowledge, the effect of encapsulation materials (alginate, pea protein, calcium ions) on the development of *C. rodentium* infection in mice have not yet been investigated.

The infection did not cause any of the animals to develop an abnormal behavior. However, the pathogen had the strongest negative effect on most of the symptoms (weight of animals, spleen size and weight, liver weight, etc.), and on histopathological changes in epithelium (epithelial hyperplasia, mitotic activity, etc.), which is supported by the findings of others, who studied effects of probiotics in murine model of infectious colitis (Johnson-Henry *et al.*, 2005; Gareau *et al.*, 2010; Costa *et al.*, 2011; Crepin *et al.*, 2016). Symptomatic data showed that both PEC (probiotic microcapsules + infection) and EC (blank microcapsules + infection) groups of mice had enlarged lymph nodes and spleens compared to the PC (probiotic powder + infection) group. However, blank microcapsules, administered to infected animals, seemed to enhance the development of the disease in cecum. Increased goblet cell depletion in cecum from infected mice within this group could be explained by the effects that alginate has on mucin-secreting cells (Forstner, 1995; Barcelo *et al.*, 2000).

Infection of mice with *C. rodentium* usually leads to the increased expression of pro-inflammatory cytokines (Higgins *et al.*, 1999; Costa *et al.*, 2011). Probiotics *L. rhamnosus* R0011 and *L. helveticus* R0052 have been previously shown to suppress the expression of pro-inflammatory cytokines in mice that have been infected with *C. rodentium* (Johnson-Henry *et al.*, 2005; Rodrigues *et al.*, 2012). In this research this effect was not observed in most of the groups. The expression of *Il17* was not decreased in infected mice receiving probiotics compared to infection controls, while the concentration of *Infγ* was not considerably high in infected animals, which might be attributed to the suppression of inflammatory response by probiotics in the PC group. *Il17* was up-regulated (in comparison to the control group) in the PEC group, indicating that the complex of encapsulation materials, probiotics and infection had a demonstrable impact on immune system, triggering inflammation. High concentrations of *Infγ* in EC and PEC groups can be attributed to the ability of alginate to induce a T_H1 immune response in mice as shown on sodium alginate-adjuvant enhanced vaccines (Dobakhti *et al.*, 2009; AbdelAllah *et al.* 2016). *Il4* was also up-regulated in the EC group in comparison to the control group, an outcome that could have been caused by calcium ions used for microcapsule crosslinking and required for the production of *Il4* by T cells as well as for the secretion of multiple cytokines by dendritic cells (Brown *et al.*, 1995; Li *et al.*, 2002; Parekh, 2010). There was a slightly higher (two-fold) increase in relative concentration of mRNA of *Tgfbβ* in infected animals compared to non-infected animals, which could be indicative of fibrosis and repair processes in the distal colon, since this cytokine plays a central role in wound healing, tissue repair and T_H17 development (Branton and Kopp, 1999; Mangan *et al.*, 2006; Penn *et al.*, 2012).

In general, infection of mice with *C. rodentium* led to the increase in the expression of pro-inflammatory cytokines, though the effect was not uniform. Probiotic administration did not significantly-impact cytokine profiles of infected animals. The highest copy numbers of *Infγ* and *Il17* mRNA were found in the distal colon of infected mice that received probiotic microcapsules (PEC), suggesting that encapsulation materials contributed to the development of inflammatory response. It has been previously suggested that sodium alginate may induce immune response in rodents, especially the expression of tumor necrosis factor alpha and interleukins 1 and 6 (Otterlei *et al.*, 1991, 1993; Son *et al.*, 2001). This effect has been linked to the interaction of alginate with the CD14 receptor on macrophages, which is similar to the activation of these cells by lipopolysaccharides, and is especially evident in case of alginates with high mannuronic acid content

(Espevik *et al.*, 1993; Otterlei *et al.*, 1993). Alginate, used in this research, consisted of ~ 61 % mannuronic acid, which could have been high enough to induce inflammation.

The interpretation of 16S rRNA analysis of mucosal communities of the distal colon remains a challenge due to the lack of apparent structure of the data, however some trends should be noted, such as a similar richness and diversity of bacterial communities in control mice and animals fed with blank capsules. These two metrics were also affected by infection – microbiome profiles became more simplified in many of the infected animals. The numbers of *Proteobacteria* were high in animals with high total average histopathological scores. This fact is supported by observations of others, where higher *Proteobacteria* counts in animals infected with *C. rodentium* were reported (Lupp *et al.*, 2007; Berry *et al.*, 2015; Shin *et al.*, 2016). In general, mucosa-associated communities of control mice, as well as mice fed with blank capsules, showed similarities and were dominated by *Firmicutes*. As reported by others (Eslinger *et al.*, 2014), the administration of pea protein can lead to the proliferation of *Bacteroides* and a decrease in the *Firmicutes/Bacteroides* ratio. Intake of alginate usually results in development of a more simplified microbiota that includes phylotypes with dominating *Firmicutes* and increased numbers of *Bacteroides*, which supports the present observation of *Firmicutes* dominating the mucosal communities of E group mice (An *et al.*, 2012; Umu *et al.*, 2015).

In my final set of experiments, PPI-alginate microcapsules were tested for their ability to protect probiotics during long-term storage. Additionally, a chitosan layer was added to the microcapsules as a way to possibly improve protective properties, and both pea protein alginate and pea protein alginate–chitosan) were used in testing of protective properties under different temperature (4°C, 22°C) and atmospheric (vacuum/no vacuum) conditions. During a storage period of 9 weeks, the cell viability resulting from both types of microcapsules was monitored and was found to be significantly-improved when microcapsules were stored at 4°C compared to 22°C. When stored at room temperature, PPI-alginate-chitosan microcapsules provided better protection for probiotic cells compared to PPI-alginate capsules. Vacuum conditions did not have any major impact on cell survival in either PPI-alginate or PPI-alginate-chitosan microcapsules stored at room temperature; however, storage of PPI-alginate-chitosan microcapsules under vacuum at 4°C led to no reduction in live bacterial numbers and only 0.3 log CFU/g reduction over 9 weeks of refrigerated storage without vacuum. Similar results of enhanced protection of cells upon addition of a chitosan layer to microcapsules were reported by Chávarri *et al.* (2010); bacterial numbers in chitosan-coated

alginate capsules decreased by 2.0 log CFU/g over 28 days of storage at 4°C. Obradović and Krunić (2015) also observed greater survival of bacteria in chitosan-coated alginate beads compared to non-coated ones during long-term refrigerated storage (4°C). Additional challenge tests (2 h in SSJ and 3 h in SIF) revealed that PPI-alginate-chitosan microcapsules provided significantly-better protection, with viable counts only decreasing by 0.7 log CFU/g compared to before-storage counts (1.5 log CFU/g for PPI-alginate capsules). After 9 weeks of storage, the same tests showed that PPI-alginate microcapsules stored with or without vacuum at room temperature provided the least protection for entrapped cells with ~ 4.1 log CFU/g decrease in viable bacterial counts, while refrigerated storage improved their performance (1.3 log CFU/g and 1.9 log CFU/g with or without vacuum, respectively).

The addition of a chitosan coating to PPI-alginate microcapsules led to significantly-improved cell viability with no loses cell viability after treatment with SSJ + SIF in samples stored under different temperature or atmosphere conditions. These results are supported by Zanjani *et al.* (2013), who reported that chitosan coating played a significant role in the protection of *Lactobacillus casei* ATCC 39392 and *Bifidobacterium bifidum* ATCC 29521 immobilized in calcium-alginate gelatinized starch microcapsules during simulated gastric- and intestinal-juice challenge tests. Similarly, chitosan-coated alginate beads provided good protection for *L. acidophilus* and *L. casei* cells during challenge tests, as reported by Krasaekoopt *et al.* (2003).

In general, according to the results of this study, the best way to preserve *L. rhamnosus* R0011 and *L. helveticus* R0052 cells over 9 weeks is by encapsulating them in chitosan-coated PPI-alginate beads along with storage at 4°C under vacuum.

8 GENERAL CONCLUSIONS

Pea protein-polysaccharide microcapsules were tested for their ability to provide sufficient protection to acid-sensitive bacteria in a series of *in vitro* experiments employing simulated conditions of human GIT. The results revealed that PPI-alginate and pea protein-*iota*-carrageenan microcapsules containing acid-sensitive *B. adolescentis* have potential for protecting these bacterial cells from the detrimental effects of the upper GIT, with subsequent release of most of the entrapped bacteria in the lower GIT. The overall cell count reduction after 2 h in SSJ at 37°C was ~ 1.0-1.5 log viable CFU/g for all types of fresh microcapsules and ~ 2.0-2.5 log CFU/g for freeze-dried capsules. The subsequent release of bacteria from the microcapsules was the highest for PPI-*iota*-carrageenan and PPI-alginate microcapsules with 70 – 74 % of the total amount of immobilized cells were released from fresh microcapsules and 75 – 79 % of immobilized cells were released from freeze-dried capsules, while PPI-gellan gum released the fewest cells, with only ~ 4.0 log CFU/g (5.3 log CFU/g for lyophilized capsules) detected following incubation in SIF. The molecular analysis of fecal samples recovered from an *in vivo* rat feeding study confirmed that PPI-alginate microcapsules are an effective tool to deliver probiotics into the lower gut.

PPI-alginate microcapsules were selected for testing in animal model of *E. coli* colitis – the *C. rodentium* infection in mice. Pathogenic infection led to marked progression of colitis, which was evident from symptomatic and histopathological data, cytokine expression and alteration of composition of mucosa-associated communities. Administration of the probiotics, *L. rhamnosus* R0011 and *L. helveticus* R0052, led to changes in most of the disease markers in cecum; however, probiotics were not able to suppress the expression of pro-inflammatory cytokines. Administration of probiotics in microcapsules did not interfere with the effect probiotics had on histopathological markers; however, EC treatment (blank microcapsules + infection) led to a set of significantly-worsened symptoms in the cecum, compared to some of the other treatments. This effect correlated with the upregulated expression of *Infy* and *IL4* in the same (EC) mouse group.

In general, the expression of cytokines was not affected by encapsulation materials.

However, a combination of infection and probiotic administration (PEC group) led to significantly-elevated levels of both *Infy* and *IL17*, suggesting a major impact on the immune system that triggered inflammation.

Studying the effects of PPI-alginate encapsulation materials in a murine model of infectious colitis was challenging due to the large, within-group diversity that accompanied almost every symptom or marker, the inability to separate the effect of each of the components of the matrix, and the lack of a definite beneficial effect of probiotics in some infected animals. However, our results did show that PPI-alginate capsule matrix worsened a set of symptoms of a disease and led to the induction of an inflammatory immune response in some infected animals. Consequently, some questions remain with respect to the utilization of PPI-alginate matrix for probiotic delivery, including possible combined pathogen-ingredient inflammation responses, species-dependent responses, and the mode of action of each component under the conditions of *C. rodentium*-induced colitis in mice.

In an attempt to further improve the protective properties of PPI-alginate capsules, a chitosan coating was added to the formulation and the effect of time and storage conditions on both capsule types investigated. Chitosan coating enhanced the survival of probiotic cells during refrigerated (4°C) storage over a 9 week period, with little difference observed in cell viability before and after challenge with simulated gastrointestinal conditions. In contrast, storage at room temperature worsened the performance (survival) of both types of capsules. Vacuum packaging did not have any significant impact on the protective properties of microcapsules stored at room temperature, but did improve cell survival at 4°C in microcapsules coated with chitosan. These results suggest that PPI-alginate microcapsules coated with chitosan have potential for preserving high viable counts in *L. rhamnosus* R0011 and *L. helveticus* R0052 during long-term refrigerated storage.

In general, a series of *in vitro* tests confirmed that PPI-alginate matrix is efficient in protecting bifidobacteria and lactobacilli from detrimental conditions of the upper GIT and assures the release of most of the encapsulated bacteria into the intestine. However, before utilization of PPI-alginate for probiotic delivery, possible pathogen-ingredient interactions and the mode of action of each component under disease conditions should be carefully studied.

9 FUTURE STUDIES

Difficulties in reproducing animal studies data suggest the need for the search for alternatives to investigate the effects that encapsulation materials and probiotics themselves might have on the GIT. One of the options that might be utilized is that of epithelial cell lines, such as HEp-2, to observe the action of pathogens, probiotics, their possible interactions and the effects encapsulation matrices might have on the system. Such cell-line systems are much cheaper, easier to reproduce and maintain (than animals), and might better allow elucidation (using gene expression analyses) of the impact of single components, such as pea protein and sodium alginate. However, this approach does not permit accounting for many of the factors that act *in vivo*, such as the effect of complex bacterial communities, immune system effects, etc. In this research, the specific strain of a pathogen and a type of cell line used did not produce relevant results, which, however, does not exclude this line of inquiry and leaves room for repeating experiments with other cell lines and probiotic and pathogen strains.

PPI-alginate microcapsules could also be tested in a different animal model of a disease *in vivo*, in conjunction with using a larger group of animal subjects. The outcome of this could yield several benefits, including improved reliability and reproducibility of results. Furthermore, the interpretation of results of this type of analyses would be easier, if the probiotics used for the treatment of infection were to have a stronger direct or indirect antagonistic activity, such as the ability to compete for attachment sites, against the pathogen along with a more predictable mode of action.

Capsule design is another area where multiple experiments could be conducted in order to improve their ability to deliver viable probiotics to specific regions of the GIT. There have been many designs suggested and tested *in vitro*; however, very few have been studied in animal models. Based on the results of this research chitosan coating provides enhanced protection to lactobacilli strains; however, its effects on GIT has not been fully described yet, therefore it is essential to test pea protein isolate alginate-chitosan microcapsules *in vivo*. Additionally, the effect of each of the

components of encapsulation matrix on the animal GIT should be thoroughly investigated. More research is also needed on the interaction of microencapsulated materials with different food matrices and modifying the chemistry of microcapsule matrices for targeted release of probiotic bacteria in specific areas of the GIT.

10 REFERENCES

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11 APPENDIX

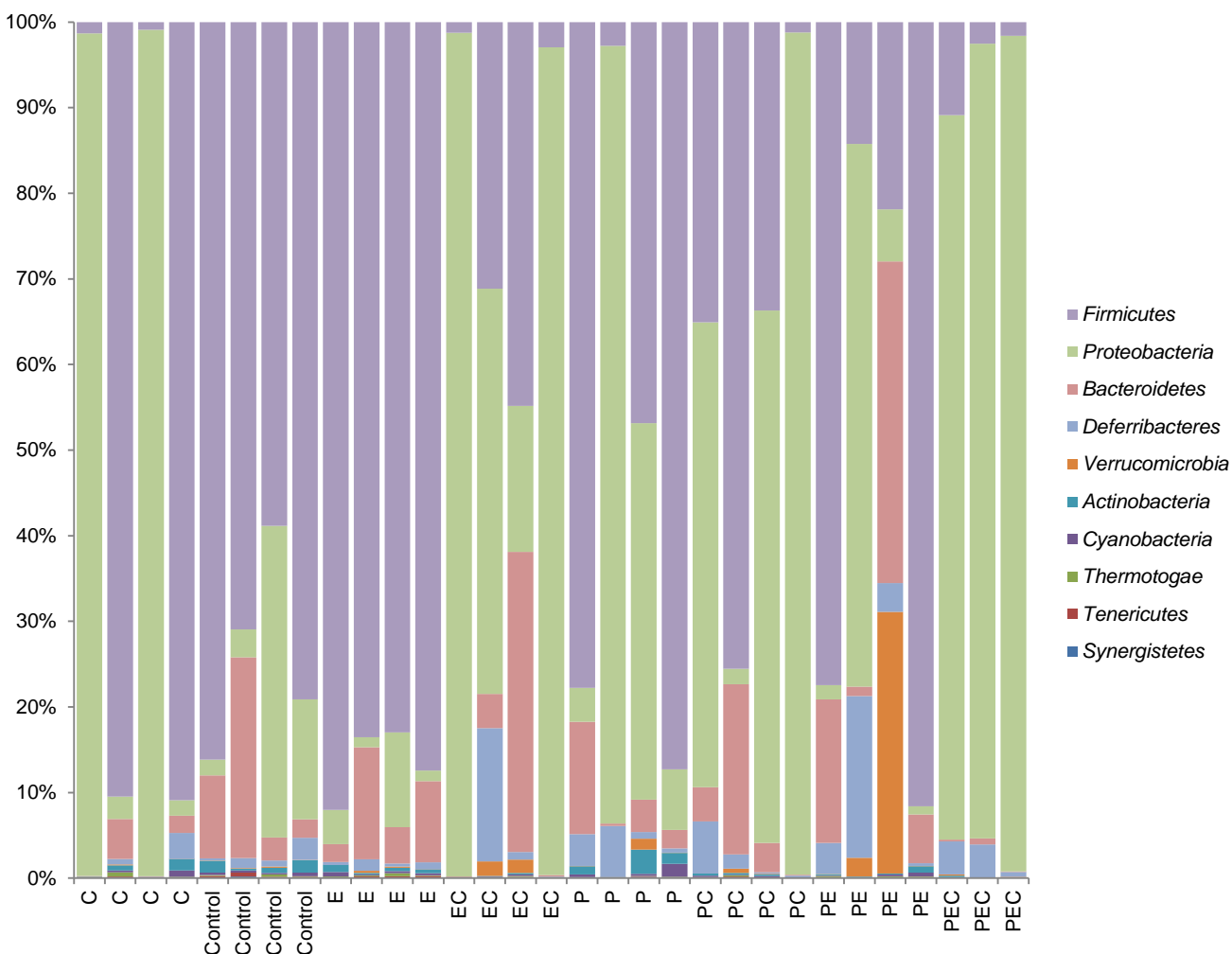


Figure A.1. Composition of mucosa-associated community (10 dominant phyla) of distal colon of mice challenged with *C. rodentium* and/or treated with encapsulated or non-encapsulated probiotics, as sequenced on Illumina MiSeq and calculated using QIIME. The y-axis shows relative abundance. Separate samples (mice) are shown on x-axis. Group abbreviations key: P – probiotics, C – *C. rodentium* infection, E – encapsulation.

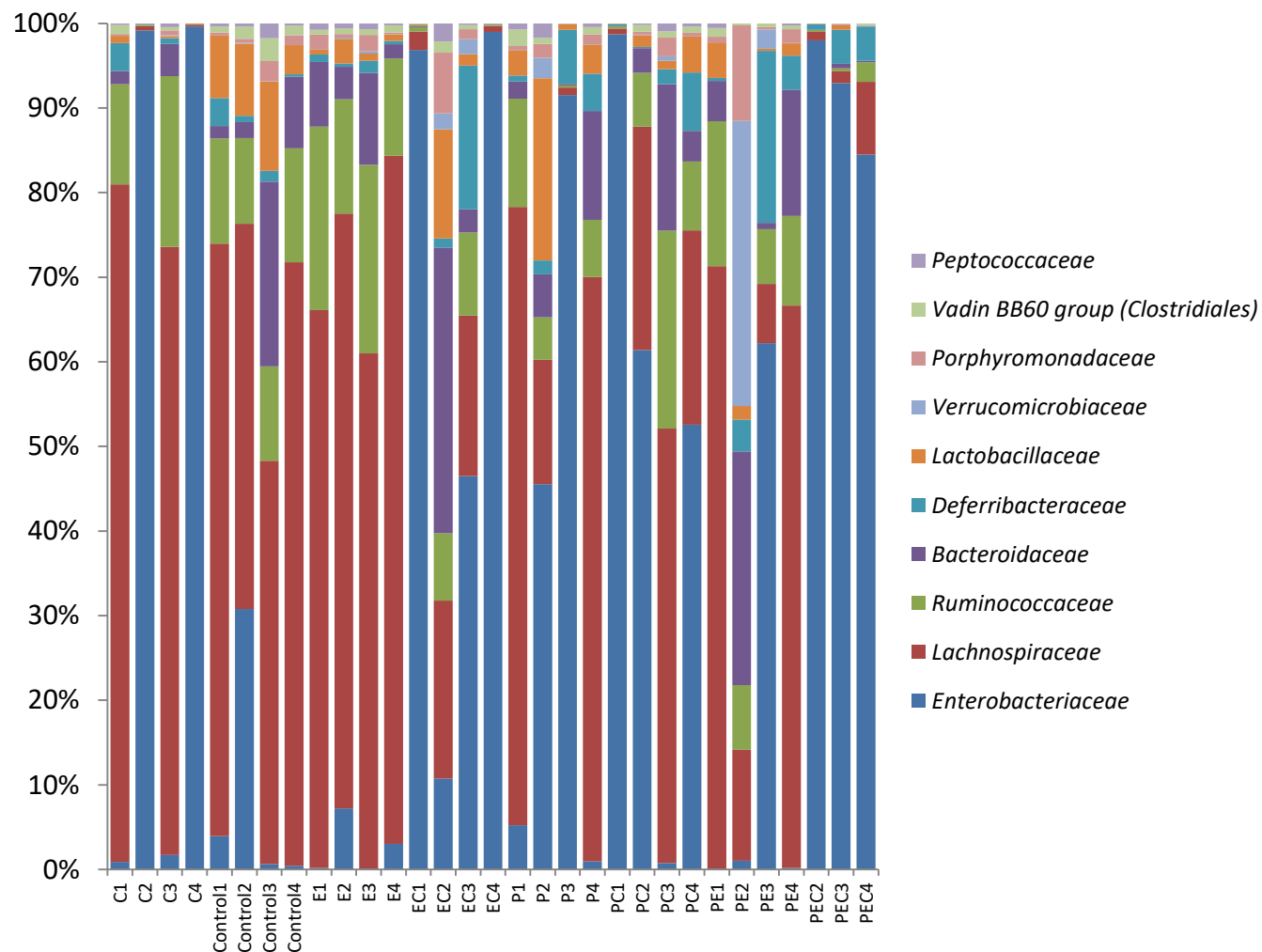


Figure A.2. Composition of mucosa-associated community (10 dominant families) of distal colon of mice challenged with *C. rodentium* and/or treated with encapsulated or non-encapsulated probiotics, as sequenced on Illumina MiSeq and calculated using QIIME. The y-axis shows relative abundance. Separate samples (mice) are shown on x-axis. Group abbreviations key: P – probiotics, C – *C. rodentium* infection, E – encapsulation.